

STANNIOCALCIN POLYNUCLEOTIDES, POLYPEPTIDES, AND METHODS BASED THEREON

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a divisional of U.S. Application No. 09/840,989, filed April 25, 2001, which is a continuation-in-part of International Application No. PCT/US00/29432, filed October 26, 2000, which claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 60/161,740, filed October 27, 1999. International Application No. PCT/US00/29432 and U.S. Provisional Application No. 60/161,740 are hereby incorporated by reference in their entirety.

FIELD OF THE INVENTION

[0002] The present invention relates to human Stanniocalcin ("STC") polynucleotides, polypeptides, and other Stanniocalcin compositions and to novel methods based thereon. In a specific embodiment, the Stanniocalcin compositions of the invention are used to treat or protect neural cells. Moreover, the present invention relates to vectors, host cells, antibodies, and recombinant and synthetic methods for producing the Stanniocalcin compositions of the invention. Also provided are diagnostic methods for detecting or prognosing diseases, disorders, damage or injury, associated with alterations of the Stanniocalcin compositions of the invention, and to therapeutic methods for treating such diseases, disorders, damage or injury

BACKGROUND OF THE INVENTION

[0003] Fish stanniocalcin is synthesized in a specialized organ adjacent to the kidney called the corpuscles of Stannius (Stannius, H., *Über nebenniere bei knochenfischen*, *Arch Anat Physiol.*, 6:97-101 (1839)). Elevated levels of calcium in plasma is a major stimulus for secretion of stanniocalcin (Wagner et al., *Mol Cell Endocrinol.*, 62:31-39 (1989); Wagner et al., *Mol Cell Endocrinol.*, 79:129-38 (1991)). Fish stanniocalcin regulates calcium and phosphate homeostasis by acting on the gills to lower the calcium

uptake on the kidney and thereby increase phosphate re-absorption, and by acting on the gut to inhibit intestinal calcium transport (Fenwick *et al.*, *J Exp. Zool.*, 188:125-131 (1974); Lafeber *et al.*, *Am J. Physiol.*, 254:R891-96 (1988); Lu *et al.*, *Am J. Physiol.*, 267:R1356-62 (1994); Sundell *et al.*, *J Comp. Physiol. [B]*, 162:489-95 (1992)). Stanniocalcin's regulation of calcium phosphate homeostasis protects against hypercalcemia.

[0004] Recently the cDNAs for human and mouse stanniocalcin were cloned (Chang *et al.*, *Mol Cell Endocrinol.*, 112:241-47 (1995); Chang *et al.*, *Mol Cell Endocrinol.*, 124:185-87 (1996); U.S. Patent Nos. 5,837,498 and 5,877,290). Human STANNIOCALCIN shares 60% identity and 80% similarity with fish stanniocalcin. Infusion of recombinant human Stanniocalcin into rats has been reported to reduce the renal excretion of phosphate (Wagner *et al.*, *J. Bone Miner Res.*, 12:165-171 (1997)). Further, it has been reported that addition of human Stanniocalcin to the serosal surface of rat or pig duodenal mucosa reduced the net absorption of calcium and increased the uptake of phosphate (Madsen *et al.*, *Am J. Physiol.*, 274:G96-102 (1998)).

[0005] Induced terminal differentiation of Paju cells, a human neural crest-derived cell, has been reported to strongly up-regulate the expression of STC. Further, the constitutive expression of Stanniocalcin has been reported to be restricted to mature neurons in human and mouse brain (Zhang *et al.*, *Am J Pathol.*, 153:439-45 (1998)).

[0006] Cerebral neurons are highly vulnerable to tissue ischemia. Mobilization and influx of calcium has long been considered a major mechanism of ischemic cell death (Seisjo *et al.*, *J Cereb Blood Flow Metab.*, 1:155-85 (1981); Seisjo *et al.*, *J Cereb Blood Flow Metab.*, 9:127-40 (1981); Choi *et al.*, *Trends Neurosci.*, 18:58-60 (1995); Kristian *et al.*, *Stroke*, 29:705-18 (1998)). Histochemical stainings have revealed prominent Stanniocalcin expression in the pyramidal cells of the cerebral cortex and hippocampus, and in the Purkinje cells of the cerebellum, (i.e. brain neurons known to be highly sensitive to ischemia) (see, e.g., Zhang *et al.*, *Am. J. Pathol.* 153:439-445 (1998) and Seisje *et al.*, *J. Cereb. Blood Flow Metab.* 1:155-185 (1981)).

[0007] There is a need for method(s) of protecting neural cells from damage and/or injury from the damaging effects of hypoxic conditions. Such methods are useful for treating, preventing the damaging effects of hypoxia brought about by such events as

infarction, stroke, and heart attack. Citation of references herein above shall not be construed as an admission that such references are prior art to the present invention.

SUMMARY OF THE INVENTION

[0008] The present invention relates to human stanniocalcin (STC) polynucleotides, polypeptides, and other Stanniocalcin compositions and to novel method based therein. In a specific embodiment, the Stanniocalcin compositions of the invention are used to treat or protect neural cells. Moreover, the present invention relates to vectors, host cells, antibodies, and recombinant and synthetic methods for producing the Stanniocalcin compositions of the invention. Also provided are diagnostic methods for detecting or prognosing diseases, disorders, damage or injury, associated with alterations of the Stanniocalcin compositions of the invention, and to therapeutic methods for treating such diseases, disorders, damage or injury.

BRIEF DESCRIPTION OF THE DRAWINGS

[0009] Figures 1A-C show the nucleotide sequence (SEQ ID NO:1) and the deduced amino acid sequence (SEQ ID NO:2) of human Stanniocalcin.

[0010] Figure 2 shows the regions of identity between the amino acid sequence of human Stanniocalcin protein (SEQ ID NO:2) and the translation product of the stanniocalcin protein from the coho salmon (*Oncorhynchus kisutch*) (SEQ ID NO:3), determined by BLAST analysis. Identical amino acids between the two polypeptides and conservative amino acid substitutions are shown. By examining the regions of amino acids that are identical and/or conservatively substituted, the skilled artisan can readily identify conserved domains between the two polypeptides. These conserved domains are preferred embodiments of the present invention.

[0011] Figure 3 shows an analysis of the human Stanniocalcin amino acid sequence (SEQ ID NO:2). Alpha, beta, turn and coil regions; hydrophilicity and hydrophobicity; amphipathic regions; flexible regions; antigenic index and surface probability are shown, and all were generated using the default settings of the recited computer programs. In the

“Antigenic Index or Jameson-Wolf” graph, the positive peaks indicate locations of the highly antigenic regions of the human Stanniocalcin protein, i.e., regions from which epitope-bearing peptides of the invention can be obtained. The domains defined by these graphs are contemplated by the present invention.

[0012] The data presented in Figure 3 are also represented in tabular form in Table I. The columns are labeled with the headings “Res”, “Position”, and Roman Numerals I-XIV. The column headings refer to the following features of the amino acid sequence presented in Figure 3, and Table I: “Res”: amino acid residue of SEQ ID NO:2 and Figures 1A and 1B; “Position”: position of the corresponding residue within SEQ ID NO:2 and Figures 1A and 1B; I: Alpha, Regions - Garnier-Robson; II: Alpha, Regions - Chou-Fasman; III: Beta, Regions - Garnier-Robson; IV: Beta, Regions - Chou-Fasman; V: Turn, Regions - Garnier-Robson; VI: Turn, Regions - Chou-Fasman; VII: Coil, Regions - Garnier-Robson; VIII: Hydrophilicity Plot - Kyte-Doolittle; IX: Hydrophobicity Plot - Hopp-Woods; X: Alpha, Amphipathic Regions - Eisenberg; XI: Beta, Amphipathic Regions - Eisenberg; XII: Flexible Regions - Karplus-Schulz; XIII: Antigenic Index - Jameson-Wolf; and XIV: Surface Probability Plot - Emini.

[0013] Figure 4 demonstrates that elevated extracellular calcium concentrations induce human Stanniocalcin expression in the neural cell line, Paju (See Example 1). Cells were cultivated in 5.4 mM CaCl₂ and lysed at indicated time points (1-48 hrs). Western blotting with rabbit antibodies revealed accumulation of STC. Timepoint 0 shows Western blotting of lysate from Paju cells cultivated in normocalcemic (0.7 mM) medium (control).

[0014] Figure 5 shows the effects of extracellular human Stanniocalcin on Pi uptake in Paju cells. Cells were incubated in Lockes' buffer containing 160 mM NaCl. The phosphate uptake was initiated by addition of 200 ng/ml recombinant Stanniocalcin together with 125 μ M KH₂³²PO₄ and the radioactivity measured at indicated time points. Control samples were without added STC. Data are presented as mean +/- SD. Asterisks * represent significance at p<0.05 compared with control samples (Student's T-test, n=6).

[0015] Figure 6 demonstrates that the overexpression of Stanniocalcin increases cell resistance to hypoxic insult. The ATP contents of Paju/C and Paju/STC cells treated with

300 μM CoCl_2 are shown at the indicated times. Data are presented as mean \pm SD. Asterisks * indicate significance at $p < 0.05$ compared with control samples (Student's T-test, $n=5-6$).

[0016] Figure 7 shows that overexpression of Stanniocalcin increases cell resistance to mobilization of intracellular calcium induced by treatment with thapsigargin.

[0017] **A:** Morphology of Paju/STC and Paju/C after treatment for 12 hrs. with 10 μM thapsigargin in serum-free culture medium.

[0018] **B:** Cell viability assay of Paju/C and Paju/STC cells treated with thapsigargin for indicated time periods. Data are presented as mean \pm SD. Asterisks * indicate significance at $p < 0.05$ compared to control samples (Student's T-test, $n=5$).

[0019] Figure 8 shows the immunohistochemical demonstration of Stanniocalcin in infarcted human parietal brain cortex.

[0020] **A:** Staining of a corresponding area from the contralateral hemisphere of a brain with a 15 hrs. old infarct (control).

[0021] **B:** Staining of the 'penumbra' of the damaged area in the infarcted hemisphere of the same brain.

[0022] **C:** Staining of 'penumbra' area from another brain with a 3 days old infarct. Arrows indicate staining of neuronal processes.

[0023] Figure 9 shows the immunohistochemical staining of Stanniocalcin in brains of rats subjected to experimental ischemia.

[0024] **A:** Six hrs. after induced focal brain ischemia covering the infarct core, 'penumbra' and peripheral area.

[0025] **B:, C:, D:** Larger magnifications of corresponding areas shown in A.

DETAILED DESCRIPTION

Definitions

[0026] The following definitions are provided to facilitate understanding of certain terms used throughout this specification.

[0027] In the present invention, “isolated” refers to material removed from its original environment (e.g., the natural environment if it is naturally occurring), and thus is altered “by the hand of man” from its natural state. For example, an isolated polynucleotide could be part of a vector or a composition of matter, or could be contained within a cell, and still be “isolated” because that vector, composition of matter, or particular cell is not the original environment of the polynucleotide. The term “isolated” does not refer to genomic or cDNA libraries, whole cell total or mRNA preparations, genomic DNA preparations (including those separated by electrophoresis and transferred onto blots), sheared whole cell genomic DNA preparations or other compositions where the art demonstrates no distinguishing features of the polynucleotide/sequences of the present invention.

[0028] In the present invention, a “secreted” stanniocalcin protein refers to a protein capable of being directed to the ER, secretory vesicles, or the extracellular space as a result of a signal sequence, as well as a stanniocalcin protein released into the extracellular space without necessarily containing a signal sequence. If the stanniocalcin secreted protein is released into the extracellular space, the stanniocalcin secreted protein can undergo extracellular processing to produce a “mature” stanniocalcin protein. Release into the extracellular space can occur by many mechanisms, including exocytosis and proteolytic cleavage.

[0029] As used herein, a stanniocalcin “polynucleotide” refers to a molecule having a nucleic acid sequence contained in SEQ ID NO:1 or the cDNA contained within the plasmid deposited with the ATCC. For example, the stanniocalcin polynucleotide can contain the nucleotide sequence of the full length cDNA sequence, including the 5' and 3' untranslated sequences, the coding region, with or without the signal sequence, the secreted protein coding region, as well as fragments, epitopes, domains, and variants of the nucleic acid sequence. Moreover, as used herein, a stanniocalcin “polypeptide” refers to a molecule having the translated amino acid sequence generated from the polynucleotide as broadly defined.

[0030] In specific embodiments, the polynucleotides of the invention are less than 300 kb, 200 kb, 100 kb, 50 kb, 15 kb, 10 kb, or 7.5 kb in length. In a further embodiment, polynucleotides of the invention comprise at least 15 contiguous nucleotides of stanniocalcin coding sequence, but do not comprise all or a portion of any stanniocalcin intron. In another embodiment, the nucleic acid comprising stanniocalcin coding sequence

does not contain coding sequences of a genomic flanking gene (i.e., 5' or 3' to the stanniocalcin gene in the genome).

[0031] In the present invention, the full length stanniocalcin sequence identified as SEQ ID NO:1 was generated by overlapping sequences of the deposited plasmid (contig analysis). A representative plasmid containing all or most of the sequence for SEQ ID NO:1 was deposited with the American Type Culture Collection ("ATCC") on January 25, 1994, and was given the ATCC Deposit Number 75652. The ATCC is located at 10801 University Boulevard, Manassas, VA 20110-2209, USA. The ATCC deposit was made pursuant to the terms of the Budapest Treaty on the international recognition of the deposit of microorganisms for purposes of patent procedure.

[0032] A stanniocalcin "polynucleotide" also includes those polynucleotides capable of hybridizing, under stringent hybridization conditions, to sequences contained in SEQ ID NO:1, the complement thereof, or the cDNA within the deposited plasmid. "Stringent hybridization conditions" refers to an overnight incubation at 42 degree C in a solution comprising 50% formamide, 5x SSC (750 mM NaCl, 75 mM sodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 μ g/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65 degree C.

[0033] Also contemplated are nucleic acid molecules that hybridize to the stanniocalcin polynucleotides at moderately high stringency hybridization conditions. Changes in the stringency of hybridization and signal detection are primarily accomplished through the manipulation of formamide concentration (lower percentages of formamide result in lowered stringency); salt conditions, or temperature. For example, moderately high stringency conditions include an overnight incubation at 37 degree C in a solution comprising 6X SSPE (20X SSPE = 3M NaCl; 0.2M NaH_2PO_4 ; 0.02M EDTA, pH 7.4), 0.5% SDS, 30% formamide, 100 μ g/ml salmon sperm blocking DNA; followed by washes at 50 degree C with 1XSSPE, 0.1% SDS. In addition, to achieve even lower stringency, washes performed following stringent hybridization can be done at higher salt concentrations (e.g. 5X SSC).

[0034] Note that variations in the above conditions may be accomplished through the inclusion and/or substitution of alternate blocking reagents used to suppress background in hybridization experiments. Typical blocking reagents include Denhardt's reagent,

BLOTTO, heparin, denatured salmon sperm DNA, and commercially available proprietary formulations. The inclusion of specific blocking reagents may require modification of the hybridization conditions described above, due to problems with compatibility.

[0035] Of course, a polynucleotide which hybridizes only to polyA⁺ sequences (such as any 3' terminal polyA⁺ tract of a cDNA shown in the sequence listing), or to a complementary stretch of T (or U) residues, would not be included in the definition of "polynucleotide," since such a polynucleotide would hybridize to any nucleic acid molecule containing a poly (A) stretch or the complement thereof (e.g., practically any double-stranded cDNA plasmid).

[0036] The stanniocalcin polynucleotide can be composed of any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. For example, stanniocalcin polynucleotides can be composed of single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, the stanniocalcin polynucleotides can be composed of triple-stranded regions comprising RNA or DNA or both RNA and DNA. Stanniocalcin polynucleotides may also contain one or more modified bases or DNA or RNA backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications can be made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically, or metabolically modified forms.

[0037] Stanniocalcin polypeptides can be composed of amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres, and may contain amino acids other than the 20 gene-encoded amino acids. The stanniocalcin polypeptides may be modified by either natural processes, such as posttranslational processing, or by chemical modification techniques which are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. Modifications can occur anywhere in the stanniocalcin polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given stanniocalcin polypeptide. Also, a

given stanniocalcin polypeptide may contain many types of modifications. Stanniocalcin polypeptides may be branched, for example, as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched, and branched cyclic stanniocalcin polypeptides may result from posttranslation natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, pegylation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. (See, for instance, *PROTEINS - STRUCTURE AND MOLECULAR PROPERTIES*, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York (1993); *POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS*, B. C. Johnson, Ed., Academic Press, New York, pgs. 1-12 (1983); Seifter et al., *Meth Enzymol* 182:626-46 (1990); Rattan et al., *Ann NY Acad Sci* 663:48-62 (1992).)

[0038] “SEQ ID NO:1” refers to a stanniocalcin polynucleotide sequence while “SEQ ID NO:2” refers to a stanniocalcin polypeptide sequence.

[0039] The Stanniocalcin polypeptides of the invention can be assayed for functional activity (e.g. biological activity) by using or routinely modifying assays known in the art, as well as assays described herein. Specifically, one of skill in the art may routinely assay Stanniocalcin polypeptides (including fragments and variants) of the invention for activity using assays as described in Examples 1, 15 and 19.

[0040] A stanniocalcin polypeptide “having biological activity” refers to polypeptides exhibiting activity similar, but not necessarily identical to, an activity of a stanniocalcin polypeptide (e.g., the ability to protect neural cells from injury associated with hypoxia), including mature forms, as measured in a particular biological assay, with or without dose dependency. In the case where dose dependency does exist, it need not be identical to that of the stanniocalcin polypeptide, but rather substantially similar to the dose-dependence in a given activity as compared to the stanniocalcin polypeptide (i.e., the candidate

polypeptide will exhibit greater activity or not more than about 25-fold less and, preferably, not more than about tenfold less activity, and most preferably, not more than about three-fold less activity relative to the stanniocalcin polypeptide.)

Stanniocalcin Polynucleotides and Polypeptides

[0041] Plasmid HLFBE10 was isolated from a human early stage lung cDNA library. This plasmid contains the entire coding region identified as SEQ ID NO:2. The deposited plasmid contains a cDNA having a total of approximately 1283 nucleotides, which encodes a predicted open reading frame of 247 amino acid residues. (See Figures 1A-C.) The open reading frame begins at a N-terminal methionine located at nucleotide position 45, and ends at a stop codon at nucleotide position 788. The predicted molecular weight of the stanniocalcin protein should be about 27.6 kDa.

[0042] Subsequent Northern analysis also showed stanniocalcin expression in stromal cells from thymus and bone marrow. Using BLAST analysis, SEQ ID NO:2 was originally found to be homologous to stanniocalcin from *Anguilla australis*.

[0043] The stanniocalcin nucleotide sequence identified as SEQ ID NO:1 was assembled from partially homologous (“overlapping”) sequences obtained from the deposited plasmid. The overlapping sequences were assembled into a single contiguous sequence of high redundancy resulting in a final sequence identified as SEQ ID NO:1.

[0044] Therefore, SEQ ID NO:1 and the translated SEQ ID NO:2 are sufficiently accurate and otherwise suitable for a variety of uses well known in the art and described further below. For instance, SEQ ID NO:1 is useful for designing nucleic acid hybridization probes that will detect nucleic acid sequences contained in SEQ ID NO:1 or the cDNA contained in the deposited plasmid. These probes will also hybridize to nucleic acid molecules in biological samples, thereby enabling a variety of forensic and diagnostic methods of the invention. Similarly, polypeptides identified from SEQ ID NO:2 may be used to generate antibodies which bind specifically to stanniocalcin.

[0045] Nevertheless, DNA sequences generated by sequencing reactions can contain sequencing errors. The errors exist as misidentified nucleotides, or as insertions or deletions of nucleotides in the generated DNA sequence. The erroneously inserted or deleted nucleotides cause frame shifts in the reading frames of the predicted amino acid sequence. In these cases, the predicted amino acid sequence diverges from the actual

amino acid sequence, even though the generated DNA sequence may be greater than 99.9% identical to the actual DNA sequence (for example, one base insertion or deletion in an open reading frame of over 1000 bases).

[0046] Accordingly, for those applications requiring precision in the nucleotide sequence or the amino acid sequence, the present invention provides not only the generated nucleotide sequence identified as SEQ ID NO:1 and the predicted translated amino acid sequence identified as SEQ ID NO:2, but also a sample of plasmid cDNA containing a human cDNA of stanniocalcin deposited with the ATCC. The nucleotide sequence of the deposited stanniocalcin plasmid can readily be determined by sequencing the deposited plasmid in accordance with known methods. The predicted stanniocalcin amino acid sequence can then be verified from such deposits. Moreover, the amino acid sequence of the protein encoded by the deposited plasmid can also be directly determined by peptide sequencing or by expressing the protein in a suitable host cell containing the deposited human stanniocalcin cDNA, collecting the protein, and determining its sequence.

[0047] The present invention also relates to the stanniocalcin gene corresponding to SEQ ID NO:1, SEQ ID NO:2, or the deposited plasmid. The stanniocalcin gene can be isolated in accordance with known methods using the sequence information disclosed herein. Such methods include preparing probes or primers from the disclosed sequence and identifying or amplifying the stanniocalcin gene from appropriate sources of genomic material.

[0048] Also provided in the present invention are species homologs of stanniocalcin. Species homologs may be isolated and identified by making suitable probes or primers from the sequences provided herein and screening a suitable nucleic acid source for the desired homologue.

[0049] The stanniocalcin polypeptides can be prepared in any suitable manner. Such polypeptides include isolated naturally occurring polypeptides, recombinantly produced polypeptides, synthetically produced polypeptides, or polypeptides produced by a combination of these methods. Means for preparing such polypeptides are well understood in the art.

[0050] The stanniocalcin polypeptides may be in the form of the secreted protein, including the mature form, or may be a part of a larger protein, such as a fusion protein

(see below). It is often advantageous to include an additional amino acid sequence which contains secretory or leader sequences, pro-sequences, sequences which aid in purification, such as multiple histidine residues, or an additional sequence for stability during recombinant production.

[0051] Stanniocalcin polypeptides and polynucleotides (and agonists or antagonists thereof) that may be used according to the methods of the present invention are further described in United States Patent Nos. 5,837,498 and 5,877,290 (the contents of which are herein incorporated by reference in their entireties).

[0052] Stanniocalcin polypeptides are preferably provided in an isolated form, and preferably are substantially purified. A recombinantly produced version of a stanniocalcin polypeptide, including the secreted polypeptide, can be substantially purified by the one-step method described in Smith and Johnson, *Gene* 67:31-40 (1988). Stanniocalcin polypeptides also can be purified from natural or recombinant sources using antibodies of the invention raised against the stanniocalcin protein in methods which are well known in the art.

Polynucleotide and Polypeptide Variants

[0053] “Variant” refers to a polynucleotide or polypeptide differing from the stanniocalcin polynucleotide or polypeptide of the present invention, but retaining essential properties thereof. Generally, variants are overall closely similar, and, in many regions, identical to the stanniocalcin polynucleotide or polypeptide of the present invention.

[0054] By a polynucleotide having a nucleotide sequence at least, for example, 95% “identical” to a reference nucleotide sequence of the present invention, it is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence encoding the stanniocalcin polypeptide. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted

into the reference sequence. The query sequence may be an entire sequence shown of SEQ ID NO:1, the ORF (open reading frame), or any fragment specified as described herein.

[0055] Other methods of determining and defining whether any particular nucleic acid molecule or polypeptide is at least 90%, 95%, 96%, 97%, 98% or 99% identical to a nucleotide sequence of the present invention can be determined conventionally using known computer programs. A preferred method for determining the best overall match between a query sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, can be determined using the FASTDB computer program based on the algorithm of Brutlag et al. (Comp. App. Biosci. (1990) 6:237-245.) In a sequence alignment the query and subject sequences are both DNA sequences. An RNA sequence can be compared by converting U's to T's. The result of said global sequence alignment is in percent identity. Preferred parameters used in a FASTDB alignment of DNA sequences to calculate percent identity are: Matrix=Unitary, k-tuple=4, Mismatch Penalty=1, Joining Penalty=30, Randomization Group Length=0, Cutoff Score=1, Gap Penalty=5, Gap Size Penalty 0.05, Window Size=500 or the length of the subject nucleotide sequence, whichever is shorter.

[0056] If the subject sequence is shorter than the query sequence because of 5' or 3' deletions, not because of internal deletions, a manual correction must be made to the results. This is because the FASTDB program does not account for 5' and 3' truncations of the subject sequence when calculating percent identity. For subject sequences truncated at the 5' or 3' ends, relative to the query sequence, the percent identity is corrected by calculating the number of bases of the query sequence that are 5' and 3' of the subject sequence, which are not matched/aligned, as a percent of the total bases of the query sequence. Whether a nucleotide is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This corrected score is what is used for the purposes of the present invention. Only bases outside the 5' and 3' bases of the subject sequence, as displayed by the FASTDB alignment, which are not matched/aligned with the query sequence, are calculated for the purposes of manually adjusting the percent identity score.

[0057] For example, a 90 base subject sequence is aligned to a 100 base query sequence to determine percent identity. The deletions occur at the 5' end of the subject sequence and therefore, the FASTDB alignment does not show a matched/alignment of the first 10 bases at 5' end. The 10 unpaired bases represent 10% of the sequence (number of bases at the 5' and 3' ends not matched/total number of bases in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 bases were perfectly matched the final percent identity would be 90%. In another example, a 90 base subject sequence is compared with a 100 base query sequence. This time the deletions are internal deletions so that there are no bases on the 5' or 3' of the subject sequence which are not matched/aligned with the query. In this case the percent identity calculated by FASTDB is not manually corrected. Once again, only bases 5' and 3' of the subject sequence which are not matched/aligned with the query sequence are manually corrected for. No other manual corrections are made for the purposes of the present invention.

[0058] By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a query amino acid sequence of the present invention, it is intended that the amino acid sequence of the subject polypeptide is identical to the query sequence except that the subject polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the query amino acid sequence. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a query amino acid sequence, up to 5% of the amino acid residues in the subject sequence may be inserted, deleted, (indels) or substituted with another amino acid. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

[0059] As a practical matter, whether any particular polypeptide is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequences shown in SEQ ID NO:2 or to the amino acid sequence encoded by the deposited cDNA plasmid can be determined conventionally using known computer programs. A preferred method for determining the best overall match between a query sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, can be

determined using the FASTDB computer program based on the algorithm of Brutlag et al. (Comp. App. Biosci. (1990) 6:237-45). In a sequence alignment the query and subject sequences are either both nucleotide sequences or both amino acid sequences. The result of said global sequence alignment is in percent identity. Preferred parameters used in a FASTDB amino acid alignment are: Matrix=PAM 0, k-tuple=2, Mismatch Penalty=1, Joining Penalty=20, Randomization Group Length=0, Cutoff Score=1, Window Size=sequence length, Gap Penalty=5, Gap Size Penalty=0.05, Window Size=500 or the length of the subject amino acid sequence, whichever is shorter.

[0060] If the subject sequence is shorter than the query sequence due to N- or C-terminal deletions, not because of internal deletions, a manual correction must be made to the results. This is because the FASTDB program does not account for N- and C-terminal truncations of the subject sequence when calculating global percent identity. For subject sequences truncated at the N- and C-termini, relative to the query sequence, the percent identity is corrected by calculating the number of residues of the query sequence that are N- and C-terminal of the subject sequence, which are not matched/aligned with a corresponding subject residue, as a percent of the total bases of the query sequence. Whether a residue is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This final percent identity score is what is used for the purposes of the present invention. Only residues to the N- and C-termini of the subject sequence, which are not matched/aligned with the query sequence, are considered for the purposes of manually adjusting the percent identity score. That is, only query residue positions outside the farthest N- and C-terminal residues of the subject sequence.

[0061] For example, a 90 amino acid residue subject sequence is aligned with a 100 residue query sequence to determine percent identity. The deletion occurs at the N-terminus of the subject sequence and therefore, the FASTDB alignment does not show a matching/alignment of the first 10 residues at the N-terminus. The 10 unpaired residues represent 10% of the sequence (number of residues at the N- and C- termini not matched/total number of residues in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 residues were perfectly matched the final percent identity would be 90%. In another example, a 90

residue subject sequence is compared with a 100 residue query sequence. This time the deletions are internal deletions so there are no residues at the N- or C-termini of the subject sequence which are not matched/aligned with the query. In this case the percent identity calculated by FASTDB is not manually corrected. Once again, only residue positions outside the N- and C-terminal ends of the subject sequence, as displayed in the FASTDB alignment, which are not matched/aligned with the query sequence are manually corrected for. No other manual corrections are made for the purposes of the present invention.

[0062] The stanniocalcin variants may contain alterations in the coding regions, non-coding regions, or both. Especially preferred are polynucleotide variants containing alterations which produce silent substitutions, additions, or deletions, but do not alter the properties or activities of the encoded polypeptide. Nucleotide variants produced by silent substitutions due to the degeneracy of the genetic code are preferred. Moreover, variants in which 5-10, 1-5, or 1-2 amino acids are substituted, deleted, or added in any combination are also preferred. stanniocalcin polynucleotide variants can be produced for a variety of reasons, e.g., to optimize codon expression for a particular host (change codons in the human mRNA to those preferred by a bacterial host such as *E. coli*).

[0063] Naturally occurring stanniocalcin variants are called “allelic variants,” and refer to one of several alternate forms of a gene occupying a given locus on a chromosome of an organism. (Genes II, Lewin, B., ed., John Wiley & Sons, New York (1985).) These allelic variants can vary at either the polynucleotide and/or polypeptide level. Alternatively, non-naturally occurring variants may be produced by mutagenesis techniques or by direct synthesis.

[0064] Using known methods of protein engineering and recombinant DNA technology, variants may be generated to improve or alter the characteristics of the stanniocalcin polypeptides. For instance, one or more amino acids can be deleted from the N-terminus or C-terminus of the secreted protein without substantial loss of biological function. The authors of Ron et al., *J. Biol. Chem.* 268:2984-88 (1993), reported variant KGF proteins having heparin binding activity even after deleting 3, 8, or 27 amino-terminal amino acid residues. Similarly, Interferon gamma exhibited up to ten times higher activity after deleting 8-10 amino acid residues from the carboxy terminus of this protein. (Dobeli et al., *J. Biotechnology* 7:199-216 (1988).)

[0065] Moreover, ample evidence demonstrates that variants often retain a biological activity similar to that of the naturally occurring protein. For example, Gayle and coworkers (J. Biol. Chem 268:22105-11 (1993)) conducted extensive mutational analysis of human cytokine IL-1a. They used random mutagenesis to generate over 3,500 individual IL-1a mutants that averaged 2.5 amino acid changes per variant over the entire length of the molecule. Multiple mutations were examined at every possible amino acid position. The investigators found that “[m]ost of the molecule could be altered with little effect on either [binding or biological activity].” (See, Abstract.) In fact, only 23 unique amino acid sequences, out of more than 3,500 nucleotide sequences examined, produced a protein that significantly differed in activity from wild-type.

[0066] Furthermore, even if deleting one or more amino acids from the N-terminus or C-terminus of a polypeptide results in modification or loss of one or more biological functions, other biological activities may still be retained. For example, the ability of a deletion variant to induce and/or to bind antibodies which recognize the secreted form will likely be retained when less than the majority of the residues of the secreted form are removed from the N-terminus or C-terminus. Whether a particular polypeptide lacking N- or C-terminal residues of a protein retains such immunogenic activities can readily be determined by routine methods described herein and otherwise known in the art.

[0067] Thus, the invention further includes stanniocalcin polypeptide variants which show substantial biological activity. Such variants include deletions, insertions, inversions, repeats, and substitutions selected according to general rules known in the art so as have little effect on activity.

[0068] The present application is directed to nucleic acid molecules at least 90%, 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequences disclosed herein, (e.g., encoding a polypeptide having the amino acid sequence of an N and/or C terminal deletion disclosed below as m-n of SEQ ID NO:2), irrespective of whether they encode a polypeptide having stanniocalcin functional activity. This is because even where a particular nucleic acid molecule does not encode a polypeptide having stanniocalcin functional activity, one of skill in the art would still know how to use the nucleic acid molecule, for instance, as a hybridization probe or a polymerase chain reaction (PCR) primer. Uses of the nucleic acid molecules of the present invention that do not encode a polypeptide having stanniocalcin functional activity include, inter alia, (1) isolating a

stanniocalcin gene or allelic or splice variants thereof in a cDNA library; (2) in situ hybridization (e.g., "FISH") to metaphase chromosomal spreads to provide precise chromosomal location of the stanniocalcin gene, as described in Verma et al., Human Chromosomes: A Manual of Basic Techniques, Pergamon Press, New York (1988); and (3) Northern Blot analysis for detecting stanniocalcin mRNA expression in specific tissues.

[0069] Preferred, however, are nucleic acid molecules having sequences at least 90%, 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequences disclosed herein, which do, in fact, encode a polypeptide having stanniocalcin functional activity. By "a polypeptide having stanniocalcin functional activity" is intended polypeptides exhibiting activity similar, but not necessarily identical, to a functional activity of the stanniocalcin polypeptides of the present invention (e.g., complete (full-length)) stanniocalcin, and mature stanniocalcin and soluble stanniocalcin as measured, for example, in a particular immunoassay or biological assay. For example, a stanniocalcin functional activity can routinely be measured by determining the ability of a stanniocalcin polypeptide to bind a stanniocalcin ligand. Stanniocalcin functional activity may also be measured by determining the ability of a polypeptide, such as cognate ligand which is free or expressed on a cell surface, to induce cells expressing the polypeptide.

[0070] Of course, due to the degeneracy of the genetic code, one of ordinary skill in the art will immediately recognize that a large number of the nucleic acid molecules having a sequence at least 90%, 95%, 96%, 97%, 98%, or 99% identical to the nucleic acid sequence of the deposited cDNA, the nucleic acid sequence shown in Figure 1 (SEQ ID NO:1), or fragments thereof, will encode polypeptides "having stanniocalcin functional activity." In fact, since degenerate variants of any of these nucleotide sequences all encode the same polypeptide, in many instances, this will be clear to the skilled artisan even without performing the above described comparison assay. It will be further recognized in the art that, for such nucleic acid molecules that are not degenerate variants, a reasonable number will also encode a polypeptide having stanniocalcin functional activity. This is because the skilled artisan is fully aware of amino acid substitutions that are either less likely or not likely to significantly effect protein function (e.g., replacing one aliphatic amino acid with a second aliphatic amino acid), as further described below.

[0071] For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie et al., “Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions,” *Science* 247:1306-10 (1990), wherein the authors indicate there are two main strategies for studying the tolerance of an amino acid sequence to change.

[0072] The first strategy exploits the tolerance of amino acid substitutions by natural selection during the process of evolution. By comparing amino acid sequences in different species, conserved amino acids can be identified. These conserved amino acids are likely important for protein function. In contrast, the amino acid positions where substitutions have been tolerated by natural selection indicates that these positions are not critical for protein function. Thus, positions tolerating amino acid substitution could be modified while still maintaining biological activity of the protein.

[0073] The second strategy uses genetic engineering to introduce amino acid changes at specific positions of a cloned gene to identify regions critical for protein function. For example, site directed mutagenesis or alanine-scanning mutagenesis (introduction of single alanine mutations at every residue in the molecule) can be used. (Cunningham and Wells, *Science* 244:1081-85 (1989).) The resulting mutant molecules can then be tested for biological activity.

[0074] As the authors state, these two strategies have revealed that proteins are surprisingly tolerant of amino acid substitutions. The authors further indicate which amino acid changes are likely to be permissive at certain amino acid positions in the protein. For example, most buried (within the tertiary structure of the protein) amino acid residues require nonpolar side chains, whereas few features of surface side chains are generally conserved. Moreover, tolerated conservative amino acid substitutions involve replacement of the aliphatic or hydrophobic amino acids Ala, Val, Leu and Ile; replacement of the hydroxyl residues Ser and Thr; replacement of the acidic residues Asp and Glu; replacement of the amide residues Asn and Gln, replacement of the basic residues Lys, Arg, and His; replacement of the aromatic residues Phe, Tyr, and Trp, and replacement of the small-sized amino acids Ala, Ser, Thr, Met, and Gly.

[0075] For example, site directed changes at the amino acid level of stanniocalcin can be made by replacing a particular amino acid with a conservative amino acid. Preferred conservative mutations include: M1 replaced with A, G, I, L, S, T, or V; L2 replaced with

A, G, I, S, T, M, or V; Q3 replaced with N; N4 replaced with Q; S5 replaced with A, G, I, L, T, M, or V; A6 replaced with G, I, L, S, T, M, or V; V7 replaced with A, G, I, L, S, T, or M; L8 replaced with A, G, I, S, T, M, or V; L9 replaced with A, G, I, S, T, M, or V; V10 replaced with A, G, I, L, S, T, or M; L11 replaced with A, G, I, S, T, M, or V; V12 replaced with A, G, I, L, S, T, or M; I13 replaced with A, G, L, S, T, M, or V; S14 replaced with A, G, I, L, T, M, or V; A15 replaced with G, I, L, S, T, M, or V; S16 replaced with A, G, I, L, T, M, or V; A17 replaced with G, I, L, S, T, M, or V; T18 replaced with A, G, I, L, S, M, or V; H19 replaced with K, or R; E20 replaced with D; A21 replaced with G, I, L, S, T, M, or V; E22 replaced with D; Q23 replaced with N; N24 replaced with Q; D25 replaced with E; S26 replaced with A, G, I, L, T, M, or V; V27 replaced with A, G, I, L, S, T, or M; S28 replaced with A, G, I, L, T, M, or V; R30 replaced with H, or K; K31 replaced with H, or R; S32 replaced with A, G, I, L, T, M, or V; R33 replaced with H, or K; V34 replaced with A, G, I, L, S, T, or M; A35 replaced with G, I, L, S, T, M, or V; A36 replaced with G, I, L, S, T, M, or V; Q37 replaced with N; N38 replaced with Q; S39 replaced with A, G, I, L, T, M, or V; A40 replaced with G, I, L, S, T, M, or V; E41 replaced with D; V42 replaced with A, G, I, L, S, T, or M; V43 replaced with A, G, I, L, S, T, or M; R44 replaced with H, or K; L46 replaced with A, G, I, S, T, M, or V; N47 replaced with Q; S48 replaced with A, G, I, L, T, M, or V; A49 replaced with G, I, L, S, T, M, or V; L50 replaced with A, G, I, S, T, M, or V; Q51 replaced with N; V52 replaced with A, G, I, L, S, T, or M; G53 replaced with A, I, L, S, T, M, or V; G55 replaced with A, I, L, S, T, M, or V; A56 replaced with G, I, L, S, T, M, or V; F57 replaced with W, or Y; A58 replaced with G, I, L, S, T, M, or V; L60 replaced with A, G, I, S, T, M, or V; E61 replaced with D; N62 replaced with Q; S63 replaced with A, G, I, L, T, M, or V; T64 replaced with A, G, I, L, S, M, or V; D66 replaced with E; T67 replaced with A, G, I, L, S, M, or V; D68 replaced with E; G69 replaced with A, I, L, S, T, M, or V; M70 replaced with A, G, I, L, S, T, or V; Y71 replaced with F, or W; D72 replaced with E; I73 replaced with A, G, L, S, T, M, or V; K75 replaced with H, or R; S76 replaced with A, G, I, L, T, M, or V; F77 replaced with W, or Y; L78 replaced with A, G, I, S, T, M, or V; Y79 replaced with F, or W; S80 replaced with A, G, I, L, T, M, or V; A81 replaced with G, I, L, S, T, M, or V; A82 replaced with G, I, L, S, T, M, or V; K83 replaced with H, or R; F84 replaced with W, or Y; D85 replaced with E; T86 replaced with A, G, I, L, S, M, or V; Q87 replaced with N; G88 replaced with A, I, L, S, T, M, or V; K89 replaced with H,

or R; A90 replaced with G, I, L, S, T, M, or V; F91 replaced with W, or Y; V92 replaced with A, G, I, L, S, T, or M; K93 replaced with H, or R; E94 replaced with D; S95 replaced with A, G, I, L, T, M, or V; L96 replaced with A, G, I, S, T, M, or V; K97 replaced with H, or R; I99 replaced with A, G, L, S, T, M, or V; A100 replaced with G, I, L, S, T, M, or V; N101 replaced with Q; G102 replaced with A, I, L, S, T, M, or V; V103 replaced with A, G, I, L, S, T, or M; T104 replaced with A, G, I, L, S, M, or V; S105 replaced with A, G, I, L, T, M, or V; K106 replaced with H, or R; V107 replaced with A, G, I, L, S, T, or M; F108 replaced with W, or Y; L109 replaced with A, G, I, S, T, M, or V; A110 replaced with G, I, L, S, T, M, or V; I111 replaced with A, G, L, S, T, M, or V; R112 replaced with H, or K; R113 replaced with H, or K; S115 replaced with A, G, I, L, T, M, or V; T116 replaced with A, G, I, L, S, M, or V; F117 replaced with W, or Y; Q118 replaced with N; R119 replaced with H, or K; M120 replaced with A, G, I, L, S, T, or V; I121 replaced with A, G, L, S, T, M, or V; A122 replaced with G, I, L, S, T, M, or V; E123 replaced with D; V124 replaced with A, G, I, L, S, T, or M; Q125 replaced with N; E126 replaced with D; E127 replaced with D; Y129 replaced with F, or W; S130 replaced with A, G, I, L, T, M, or V; K131 replaced with H, or R; L132 replaced with A, G, I, S, T, M, or V; N133 replaced with Q; V134 replaced with A, G, I, L, S, T, or M; S136 replaced with A, G, I, L, T, M, or V; I137 replaced with A, G, L, S, T, M, or V; A138 replaced with G, I, L, S, T, M, or V; K139 replaced with H, or R; R140 replaced with H, or K; N141 replaced with Q; E143 replaced with D; A144 replaced with G, I, L, S, T, M, or V; I145 replaced with A, G, L, S, T, M, or V; T146 replaced with A, G, I, L, S, M, or V; E147 replaced with D; V148 replaced with A, G, I, L, S, T, or M; V149 replaced with A, G, I, L, S, T, or M; Q150 replaced with N; L151 replaced with A, G, I, S, T, M, or V; N153 replaced with Q; H154 replaced with K, or R; F155 replaced with W, or Y; S156 replaced with A, G, I, L, T, M, or V; N157 replaced with Q; R158 replaced with H, or K; Y159 replaced with F, or W; Y160 replaced with F, or W; N161 replaced with Q; R162 replaced with H, or K; L163 replaced with A, G, I, S, T, M, or V; V164 replaced with A, G, I, L, S, T, or M; R165 replaced with H, or K; S166 replaced with A, G, I, L, T, M, or V; L167 replaced with A, G, I, S, T, M, or V; L168 replaced with A, G, I, S, T, M, or V; E169 replaced with D; D171 replaced with E; E172 replaced with D; D173 replaced with E; T174 replaced with A, G, I, L, S, M, or V; V175 replaced with A, G, I, L, S, T, or M; S176 replaced with A, G, I, L, T, M, or V; T177 replaced with A, G, I, L, S, M, or V; I178 replaced with A, G, L,

S, T, M, or V; R179 replaced with H, or K; D180 replaced with E; S181 replaced with A, G, I, L, T, M, or V; L182 replaced with A, G, I, S, T, M, or V; M183 replaced with A, G, I, L, S, T, or V; E184 replaced with D; K185 replaced with H, or R; I186 replaced with A, G, L, S, T, M, or V; G187 replaced with A, I, L, S, T, M, or V; N189 replaced with Q; M190 replaced with A, G, I, L, S, T, or V; A191 replaced with G, I, L, S, T, M, or V; S192 replaced with A, G, I, L, T, M, or V; L193 replaced with A, G, I, S, T, M, or V; F194 replaced with W, or Y; H195 replaced with K, or R; I196 replaced with A, G, L, S, T, M, or V; L197 replaced with A, G, I, S, T, M, or V; Q198 replaced with N; T199 replaced with A, G, I, L, S, M, or V; D200 replaced with E; H201 replaced with K, or R; A203 replaced with G, I, L, S, T, M, or V; Q204 replaced with N; T205 replaced with A, G, I, L, S, M, or V; H206 replaced with K, or R; R208 replaced with H, or K; A209 replaced with G, I, L, S, T, M, or V; D210 replaced with E; F211 replaced with W, or Y; N212 replaced with Q; R213 replaced with H, or K; R214 replaced with H, or K; R215 replaced with H, or K; T216 replaced with A, G, I, L, S, M, or V; N217 replaced with Q; E218 replaced with D; Q220 replaced with N; K221 replaced with H, or R; L222 replaced with A, G, I, S, T, M, or V; K223 replaced with H, or R; V224 replaced with A, G, I, L, S, T, or M; L225 replaced with A, G, I, S, T, M, or V; L226 replaced with A, G, I, S, T, M, or V; R227 replaced with H, or K; N228 replaced with Q; L229 replaced with A, G, I, S, T, M, or V; R230 replaced with H, or K; G231 replaced with A, I, L, S, T, M, or V; E232 replaced with D; E233 replaced with D; D234 replaced with E; S235 replaced with A, G, I, L, T, M, or V; S237 replaced with A, G, I, L, T, M, or V; H238 replaced with K, or R; I239 replaced with A, G, L, S, T, M, or V; K240 replaced with H, or R; R241 replaced with H, or K; T242 replaced with A, G, I, L, S, M, or V; S243 replaced with A, G, I, L, T, M, or V; H244 replaced with K, or R; E245 replaced with D; S246 replaced with A, G, I, L, T, M, or V; A247 replaced with G, I, L, S, T, M, or V.

[0076] The resulting constructs can be routinely screened for activities or functions described throughout the specification and known in the art. Preferably, the resulting constructs have an increased stanniocalcin activity or function, while the remaining stanniocalcin activities or functions are maintained. More preferably, the resulting constructs have more than one increased stanniocalcin activity or function, while the remaining stanniocalcin activities or functions are maintained.

[0077] Besides conservative amino acid substitution, variants of the present invention include (i) substitutions with one or more of the non-conserved amino acid residues, where the substituted amino acid residues may or may not be one encoded by the genetic code, or (ii) substitutions with one or more of the amino acid residues having a substituent group, or (iii) fusion of the mature polypeptide with another compound, such as a compound to increase the stability and/or solubility of the polypeptide (for example, polyethylene glycol), (iv) fusion of the polypeptide with additional amino acids, such as, for example, an IgG Fc fusion region peptide, serum albumin (preferably human serum albumin) or a fragment thereof, or leader or secretory sequence, or a sequence facilitating purification, or (v) fusion of the polypeptide with another compound, such as albumin (including but not limited to recombinant albumin (see, e.g., U.S. Patent No. 5,876,969, issued March 2, 1999, EP Patent 0 413 622, and U.S. Patent No. 5,766,883, issued June 16, 1998, herein incorporated by reference in their entirety)). Such variant polypeptides are deemed to be within the scope of those skilled in the art from the teachings herein.

[0078] For example, stanniocalcin polypeptide variants containing amino acid substitutions of charged amino acids with other charged or neutral amino acids may produce proteins with improved characteristics, such as less aggregation. Aggregation of pharmaceutical formulations both reduces activity and increases clearance due to the aggregate's immunogenic activity. (Pinckard et al., Clin. Exp. Immunol. 2:331-340 (1967); Robbins et al., Diabetes 36: 838-845 (1987); Cleland et al., Crit. Rev. Therapeutic Drug Carrier Systems 10:307-377 (1993)).

[0079] For example, preferred non-conservative substitutions of stanniocalcin include M1 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; L2 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; Q3 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, F, W, Y, P, or C; N4 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, F, W, Y, P, or C; S5 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; A6 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; V7 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; L8 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; L9 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; V10 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; L11 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; V12 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; I13 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; S14 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; A15 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; S16

replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; A17 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; T18 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; H19 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; E20 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; A21 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; E22 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; Q23 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, F, W, Y, P, or C; N24 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, F, W, Y, P, or C; D25 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; S26 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; V27 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; S28 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; P29 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, or C; R30 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; K31 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; S32 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; R33 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; V34 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; A35 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; A36 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; Q37 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, F, W, Y, P, or C; N38 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, F, W, Y, P, or C; S39 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; A40 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; E41 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; V42 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; V43 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; R44 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; C45 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, or P; L46 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; N47 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, F, W, Y, P, or C; S48 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; A49 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; L50 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; Q51 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, F, W, Y, P, or C; V52 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; G53 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; C54 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, or P; G55 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; A56 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; F57 replaced with D, E, H, K, R, N, Q, A, G, I, L, S, T, M, V, P, or C; A58 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; C59 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, or P; L60

replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; E61 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; N62 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, F, W, Y, P, or C; S63 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; T64 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; C65 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, or P; D66 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; T67 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; D68 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; G69 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; M70 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; Y71 replaced with D, E, H, K, R, N, Q, A, G, I, L, S, T, M, V, P, or C; D72 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; I73 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; C74 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, or P; K75 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; S76 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; F77 replaced with D, E, H, K, R, N, Q, A, G, I, L, S, T, M, V, P, or C; L78 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; Y79 replaced with D, E, H, K, R, N, Q, A, G, I, L, S, T, M, V, P, or C; S80 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; A81 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; A82 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; K83 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; F84 replaced with D, E, H, K, R, N, Q, A, G, I, L, S, T, M, V, P, or C; D85 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; T86 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; Q87 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, F, W, Y, P, or C; G88 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; K89 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; A90 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; F91 replaced with D, E, H, K, R, N, Q, A, G, I, L, S, T, M, V, P, or C; V92 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; K93 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; E94 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; S95 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; L96 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; K97 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; C98 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, or P; I99 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; A100 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; N101 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, F, W, Y, P, or C; G102 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; V103 replaced with D, E, H, K, R, N, Q, F, W, Y,

P, or C; T104 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; S105 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; K106 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; V107 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; F108 replaced with D, E, H, K, R, N, Q, A, G, I, L, S, T, M, V, P, or C; L109 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; A110 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; I111 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; R112 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; R113 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; C114 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, or P; S115 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; T116 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; F117 replaced with D, E, H, K, R, N, Q, A, G, I, L, S, T, M, V, P, or C; Q118 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, F, W, Y, P, or C; R119 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; M120 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; I121 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; A122 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; E123 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; V124 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; Q125 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, F, W, Y, P, or C; E126 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; E127 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; C128 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, or P; Y129 replaced with D, E, H, K, R, N, Q, A, G, I, L, S, T, M, V, P, or C; S130 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; K131 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; L132 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; N133 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, F, W, Y, P, or C; V134 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; C135 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, or P; S136 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; I137 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; A138 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; K139 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; R140 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; N141 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, F, W, Y, P, or C; P142 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, or C; E143 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; A144 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; I145 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; T146 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or

C; E147 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; V148 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; V149 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; Q150 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, F, W, Y, P, or C; L151 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; P152 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, or C; N153 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, F, W, Y, P, or C; H154 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; F155 replaced with D, E, H, K, R, N, Q, A, G, I, L, S, T, M, V, P, or C; S156 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; N157 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, F, W, Y, P, or C; R158 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; Y159 replaced with D, E, H, K, R, N, Q, A, G, I, L, S, T, M, V, P, or C; Y160 replaced with D, E, H, K, R, N, Q, A, G, I, L, S, T, M, V, P, or C; N161 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, F, W, Y, P, or C; R162 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; L163 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; V164 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; R165 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; S166 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; L167 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; L168 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; E169 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; C170 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, or P; D171 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; E172 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; D173 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; T174 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; V175 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; S176 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; T177 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; I178 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; R179 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; D180 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; S181 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; L182 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; M183 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; E184 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; K185 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; I186 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; G187 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; P188 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, or C; N189 replaced

with D, E, H, K, R, A, G, I, L, S, T, M, V, F, W, Y, P, or C; M190 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; A191 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; S192 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; L193 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; F194 replaced with D, E, H, K, R, N, Q, A, G, I, L, S, T, M, V, P, or C; H195 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; I196 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; L197 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; Q198 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, F, W, Y, P, or C; T199 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; D200 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; H201 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; C202 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, or P; A203 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; Q204 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, F, W, Y, P, or C; T205 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; H206 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; P207 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, or C; R208 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; A209 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; D210 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; F211 replaced with D, E, H, K, R, N, Q, A, G, I, L, S, T, M, V, P, or C; N212 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, F, W, Y, P, or C; R213 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; R214 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; R215 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; T216 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; N217 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, F, W, Y, P, or C; E218 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; P219 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, or C; Q220 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, F, W, Y, P, or C; K221 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; L222 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; K223 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; V224 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; L225 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; L226 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; R227 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; N228 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, F, W, Y, P, or C; L229 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; R230 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C;

C; G231 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; E232 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; E233 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; D234 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; S235 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; P236 replaced with D, E, H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, or C; S237 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; H238 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; I239 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; K240 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; R241 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; T242 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; S243 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; H244 replaced with D, E, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; E245 replaced with H, K, R, A, G, I, L, S, T, M, V, N, Q, F, W, Y, P, or C; S246 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C; A247 replaced with D, E, H, K, R, N, Q, F, W, Y, P, or C.

[0080] The resulting constructs can be routinely screened for activities or functions described throughout the specification and known in the art. Preferably, the resulting constructs have loss of a stanniocalcin activity or function, while the remaining stanniocalcin activities or functions are maintained. More preferably, the resulting constructs have more than one loss of stanniocalcin activity or function, while the remaining stanniocalcin activities or functions are maintained.

[0081] Additionally, more than one amino acid (e.g., 2, 3, 4, 5, 6, 7, 8, 9 and 10) can be replaced with the substituted amino acids as described above (either conservative or nonconservative). The substituted amino acids can occur in the full length, mature, or proprotein form of stanniocalcin protein, as well as the N- and C- terminal deletion mutants, having the general formula m-n, listed below.

[0082] A further embodiment of the invention relates to a polypeptide which comprises the amino acid sequence of a stanniocalcin polypeptide having an amino acid sequence which contains at least one amino acid substitution, but not more than 50 amino acid substitutions, even more preferably, not more than 40 amino acid substitutions, still more preferably, not more than 30 amino acid substitutions, and still even more preferably, not more than 20 amino acid substitutions. Of course, in order of ever-increasing preference, it is highly preferable for a peptide or polypeptide to have an amino acid sequence which comprises the amino acid sequence of a stanniocalcin polypeptide, which contains at least

one, but not more than 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acid substitutions. In specific embodiments, the number of additions, substitutions, and/or deletions in the amino acid sequence of Figure 1 or fragments thereof (e.g., the mature form and/or other fragments described herein), is 1-5, 5-10, 5-25, 5-50, 10-50 or 50-150, conservative amino acid substitutions are preferable.

Polynucleotide and Polypeptide Fragments

[0083] The present invention is further directed to fragments of the isolated nucleic acid molecules described herein. By a fragment of an isolated nucleic acid molecule having, for example, the nucleotide sequence of the deposited cDNA (plasmid HLFBE10), a nucleotide sequence encoding the polypeptide sequence encoded by the deposited cDNA, a nucleotide sequence encoding the polypeptide sequence depicted in Figure 1 (SEQ ID NO:2), the nucleotide sequence shown in Figure 1 (SEQ ID NO:1), or the complementary strand thereto, is intended fragments at least 15 nt, and more preferably at least about 20 nt, still more preferably at least 30 nt, and even more preferably, at least about 40, 50, 100, 150, 200, 250, 300, 325, 350, 375, 400, 450, 500, 550, or 600 nt in length. These fragments have numerous uses that include, but are not limited to, diagnostic probes and primers as discussed herein. Of course, larger fragments, such as those of 501-1500 nt in length are also useful according to the present invention as are fragments corresponding to most, if not all, of the nucleotide sequences of the deposited cDNA (plasmid HLFBE10) or as shown in Figure 1 (SEQ ID NO:1). By a fragment at least 20 nt in length, for example, is intended fragments which include 20 or more contiguous bases from, for example, the nucleotide sequence of the deposited cDNA, or the nucleotide sequence as shown in Figure 1 (SEQ ID NO:1).

[0084] Moreover, representative examples of stanniocalcin polynucleotide fragments include, for example, fragments having a sequence from about nucleotide number 1-50, 51-100, 101-150, 151-200, 201-250, 251-300, 301-350, 351-400, 401-450, 451-500, 501-550, 551-600, 651-700, 701-750, 751-800, 800-850, 851-900, 901-950, 951-1000, 1001-1050, 1051-1100, 1101-1150, 1151-1200, 1201-1250, and 1251-1283 of SEQ ID NO:1 or the complementary strand thereto, or the cDNA contained in the deposited plasmid. In this context "about" includes the particularly recited ranges, larger or smaller by several (5, 4, 3, 2, or 1) nucleotides, at either terminus or at both termini.

[0085] Preferably, the polynucleotide fragments of the invention encode a polypeptide which demonstrates a stanniocalcin functional activity. By a polypeptide demonstrating a stanniocalcin “functional activity” is meant, a polypeptide capable of displaying one or more known functional activities associated with a full-length (complete) stanniocalcin protein. Such functional activities include, but are not limited to, biological activity (e.g., ability to protect neurons challenged by hypoxia or ischemia (as tested in vivo or in vitro, for example, by treatment with CoCl_2 or other compounds which mimic hypoxic insults on neural tissue)), antigenicity [ability to bind (or compete with a stanniocalcin polypeptide for binding) to an anti-stanniocalcin antibody], immunogenicity (ability to generate antibody which binds to a stanniocalcin polypeptide), ability to form multimers with stanniocalcin polypeptides of the invention, and ability to bind to a receptor or ligand for a stanniocalcin polypeptide.

[0086] The functional activity of stanniocalcin polypeptides, and fragments, variants derivatives, and analogs thereof, can be assayed by various methods.

[0087] For example, in one embodiment where one is assaying for the ability to bind or compete with full-length stanniocalcin polypeptide for binding to anti-stanniocalcin antibody, various immunoassays known in the art can be used, including but not limited to, competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay), “sandwich” immunoassays, immunoradiometric assays, gel diffusion precipitation reactions, immunodiffusion assays, in situ immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blots, precipitation reactions, agglutination assays (e.g., gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention.

[0088] In another embodiment, where a stanniocalcin ligand is identified, or the ability of a polypeptide fragment, variant or derivative of the invention to multimerize is being evaluated, binding can be assayed, e.g., by means well-known in the art, such as, for

example, reducing and non-reducing gel chromatography, protein affinity chromatography, and affinity blotting. See generally, Phizicky, E., et al., 1995, *Microbiol. Rev.* 59:94-123. In another embodiment, physiological correlates of stanniocalcin binding to its substrates (signal transduction) can be assayed.

[0089] In addition, assays described herein (see, e.g., Example 1) and otherwise known in the art may routinely be applied to measure the ability of stanniocalcin polypeptides and fragments, variants derivatives and analogs thereof to elicit stanniocalcin related biological activity (either in vitro or in vivo). Other methods will be known to the skilled artisan and are within the scope of the invention.

[0090] The present invention is further directed to fragments of the stanniocalcin polypeptide described herein. By a fragment of an isolated stanniocalcin polypeptide, for example, encoded by the deposited cDNA (plasmid HLFBE10), the polypeptide sequence encoded by the deposited cDNA, the polypeptide sequence depicted in Figure 1 (SEQ ID NO:2), is intended to encompass polypeptide fragments contained in SEQ ID NO:2 or encoded by the cDNA contained in the deposited plasmid. Protein fragments may be “free-standing,” or comprised within a larger polypeptide of which the fragment forms a part or region, most preferably as a single continuous region. Representative examples of polypeptide fragments of the invention, include, for example, fragments from about amino acid number 1-20, 21-40, 41-60, 61-80, 81-100, 102-120, 121-140, 141-160, 161-180, 181-200, 201-220, 221-240, or 241-247 of the coding region. Moreover, polypeptide fragments can be at least 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, or 150 amino acids in length. In this context “about” includes the particularly recited ranges, larger or smaller by several (5, 4, 3, 2, or 1) amino acids, at either extreme or at both extremes.

[0091] Even if deletion of one or more amino acids from the N-terminus of a protein results in modification or loss of one or more biological functions of the protein, other functional activities (e.g., biological activities, ability to multimerize, ability to bind stanniocalcin ligand) may still be retained. For example, the ability of shortened stanniocalcin muteins to induce and/or bind to antibodies which recognize the complete or mature forms of the polypeptides generally will be retained when less than the majority of the residues of the complete or mature polypeptide are removed from the N-terminus. Whether a particular polypeptide lacking N-terminal residues of a complete polypeptide

retains such immunologic activities can readily be determined by routine methods described herein and otherwise known in the art. It is not unlikely that a stanniocalcin mutein with a large number of deleted N-terminal amino acid residues may retain some biological or immunogenic activities. In fact, peptides composed of as few as six stanniocalcin amino acid residues may often evoke an immune response.

[0092] Accordingly, polypeptide fragments of the invention include the mature (secreted) stanniocalcin protein having a continuous series of deleted residues from the amino or the carboxy terminus, or both. For example, any number of amino acids, ranging from 1-60, can be deleted from the amino terminus of either the secreted stanniocalcin polypeptide. Similarly, any number of amino acids, ranging from 1-30, can be deleted from the carboxy terminus of the secreted stanniocalcin protein. Furthermore, any combination of the above amino and carboxy terminus deletions are preferred. Similarly, polynucleotide fragments encoding these stanniocalcin polypeptide fragments are also preferred.

[0093] Particularly, N-terminal deletions of the stanniocalcin polypeptide can be described by the general formula m-247, where m is an integer from 2 to 242, where m corresponds to the position of the amino acid residue identified in SEQ ID NO:2. More in particular, the invention provides polypeptides comprising, or alternatively consisting of, the amino acid sequence of residues of L-2 to A-247; Q-3 to A-247; N-4 to A-247; S-5 to A-247; A-6 to A-247; V-7 to A-247; L-8 to A-247; L-9 to A-247; V-10 to A-247; L-11 to A-247; V-12 to A-247; I-13 to A-247; S-14 to A-247; A-15 to A-247; S-16 to A-247; A-17 to A-247; T-18 to A-247; H-19 to A-247; E-20 to A-247; A-21 to A-247; E-22 to A-247; Q-23 to A-247; N-24 to A-247; D-25 to A-247; S-26 to A-247; V-27 to A-247; S-28 to A-247; P-29 to A-247; R-30 to A-247; K-31 to A-247; S-32 to A-247; R-33 to A-247; V-34 to A-247; A-35 to A-247; A-36 to A-247; Q-37 to A-247; N-38 to A-247; S-39 to A-247; A-40 to A-247; E-41 to A-247; V-42 to A-247; V-43 to A-247; R-44 to A-247; C-45 to A-247; L-46 to A-247; N-47 to A-247; S-48 to A-247; A-49 to A-247; L-50 to A-247; Q-51 to A-247; V-52 to A-247; G-53 to A-247; C-54 to A-247; G-55 to A-247; A-56 to A-247; F-57 to A-247; A-58 to A-247; C-59 to A-247; L-60 to A-247; E-61 to A-247; N-62 to A-247; S-63 to A-247; T-64 to A-247; C-65 to A-247; D-66 to A-247; T-67 to A-247; D-68 to A-247; G-69 to A-247; M-70 to A-247; Y-71 to A-247; D-72 to A-247; I-73 to A-247; C-74 to A-247; K-75 to A-247; S-76 to A-247; F-77 to A-247; L-78 to A-247; Y-79

to A-247; S-80 to A-247; A-81 to A-247; A-82 to A-247; K-83 to A-247; F-84 to A-247; D-85 to A-247; T-86 to A-247; Q-87 to A-247; G-88 to A-247; K-89 to A-247; A-90 to A-247; F-91 to A-247; V-92 to A-247; K-93 to A-247; E-94 to A-247; S-95 to A-247; L-96 to A-247; K-97 to A-247; C-98 to A-247; I-99 to A-247; A-100 to A-247; N-101 to A-247; G-102 to A-247; V-103 to A-247; T-104 to A-247; S-105 to A-247; K-106 to A-247; V-107 to A-247; F-108 to A-247; L-109 to A-247; A-110 to A-247; I-111 to A-247; R-112 to A-247; R-113 to A-247; C-114 to A-247; S-115 to A-247; T-116 to A-247; F-117 to A-247; Q-118 to A-247; R-119 to A-247; M-120 to A-247; I-121 to A-247; A-122 to A-247; E-123 to A-247; V-124 to A-247; Q-125 to A-247; E-126 to A-247; E-127 to A-247; C-128 to A-247; Y-129 to A-247; S-130 to A-247; K-131 to A-247; L-132 to A-247; N-133 to A-247; V-134 to A-247; C-135 to A-247; S-136 to A-247; I-137 to A-247; A-138 to A-247; K-139 to A-247; R-140 to A-247; N-141 to A-247; P-142 to A-247; E-143 to A-247; A-144 to A-247; I-145 to A-247; T-146 to A-247; E-147 to A-247; V-148 to A-247; V-149 to A-247; Q-150 to A-247; L-151 to A-247; P-152 to A-247; N-153 to A-247; H-154 to A-247; F-155 to A-247; S-156 to A-247; N-157 to A-247; R-158 to A-247; Y-159 to A-247; Y-160 to A-247; N-161 to A-247; R-162 to A-247; L-163 to A-247; V-164 to A-247; R-165 to A-247; S-166 to A-247; L-167 to A-247; L-168 to A-247; E-169 to A-247; C-170 to A-247; D-171 to A-247; E-172 to A-247; D-173 to A-247; T-174 to A-247; V-175 to A-247; S-176 to A-247; T-177 to A-247; I-178 to A-247; R-179 to A-247; D-180 to A-247; S-181 to A-247; L-182 to A-247; M-183 to A-247; E-184 to A-247; K-185 to A-247; I-186 to A-247; G-187 to A-247; P-188 to A-247; N-189 to A-247; M-190 to A-247; A-191 to A-247; S-192 to A-247; L-193 to A-247; F-194 to A-247; H-195 to A-247; I-196 to A-247; L-197 to A-247; Q-198 to A-247; T-199 to A-247; D-200 to A-247; H-201 to A-247; C-202 to A-247; A-203 to A-247; Q-204 to A-247; T-205 to A-247; H-206 to A-247; P-207 to A-247; R-208 to A-247; A-209 to A-247; D-210 to A-247; F-211 to A-247; N-212 to A-247; R-213 to A-247; R-214 to A-247; R-215 to A-247; T-216 to A-247; N-217 to A-247; E-218 to A-247; P-219 to A-247; Q-220 to A-247; K-221 to A-247; L-222 to A-247; K-223 to A-247; V-224 to A-247; L-225 to A-247; L-226 to A-247; R-227 to A-247; N-228 to A-247; L-229 to A-247; R-230 to A-247; G-231 to A-247; E-232 to A-247; E-233 to A-247; D-234 to A-247; S-235 to A-247; P-236 to A-247; S-237 to A-247; H-238 to A-247; I-239 to A-247; K-240 to A-247; R-241 to A-247; T-242 to A-247; of SEQ ID NO: 2. Polynucleotides encoding these polypeptides are also encompassed by the

invention, as are antibodies that bind one or more of these polypeptides. Moreover, fragments and variants of these polypeptides (e.g., fragments as described herein, polypeptides at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to these polypeptides and polypeptides encoded by the polynucleotide which hybridizes, under stringent conditions, to the polynucleotide encoding these polypeptides, or the complement thereof) are encompassed by the invention. Antibodies that bind these fragments and variants of the invention are also encompassed by the invention. Polynucleotides encoding these fragments and variants are also encompassed by the invention.

[0094] Also as mentioned above, even if the deletion of one or more amino acids from the C-terminus of a protein results in modification or loss of one or more biological functions of the protein, other functional activities (e.g., biological activities, ability to multimerize, ability to bind receptor) may still be retained. For example the ability of the shortened stanniocalcin mutein to induce and/or bind to antibodies which recognize the complete or mature forms of the polypeptide generally will be retained when less than the majority of the residues of the complete or mature polypeptide are removed from the C-terminus. Whether a particular polypeptide lacking C-terminal residues of a complete polypeptide retains such immunologic activities can readily be determined by routine methods described herein and otherwise known in the art. It is not unlikely that an stanniocalcin mutein with a large number of deleted C-terminal amino acid residues may retain some biological or immunogenic activities. In fact, peptides composed of as few as six stanniocalcin amino acid residues may often evoke an immune response.

[0095] Accordingly, the present invention further provides polypeptides having one or more residues deleted from the carboxy terminus of the amino acid sequence of the stanniocalcin polypeptide shown in Figure 1 (SEQ ID NO:2), as described by the general formula 1-n, where n is an integer from 7 to 246, where n corresponds to the position of amino acid residue identified in SEQ ID NO:2. More in particular, the invention provides polypeptides comprising, or alternatively consisting of, the amino acid sequence of residues of M-1 to S-246; M-1 to E-245; M-1 to H-244; M-1 to S-243; M-1 to T-242; M-1 to R-241; M-1 to K-240; M-1 to I-239; M-1 to H-238; M-1 to S-237; M-1 to P-236; M-1 to S-235; M-1 to D-234; M-1 to E-233; M-1 to E-232; M-1 to G-231; M-1 to R-230; M-1 to L-229; M-1 to N-228; M-1 to R-227; M-1 to L-226; M-1 to L-225; M-1 to V-224; M-1 to K-223; M-1 to L-222; M-1 to K-221; M-1 to Q-220; M-1 to P-219; M-1 to E-218; M-1

to N-217; M-1 to T-216; M-1 to R-215; M-1 to R-214; M-1 to R-213; M-1 to N-212; M-1 to F-211; M-1 to D-210; M-1 to A-209; M-1 to R-208; M-1 to P-207; M-1 to H-206; M-1 to T-205; M-1 to Q-204; M-1 to A-203; M-1 to C-202; M-1 to H-201; M-1 to D-200; M-1 to T-199; M-1 to Q-198; M-1 to L-197; M-1 to I-196; M-1 to H-195; M-1 to F-194; M-1 to L-193; M-1 to S-192; M-1 to A-191; M-1 to M-190; M-1 to N-189; M-1 to P-188; M-1 to G-187; M-1 to I-186; M-1 to K-185; M-1 to E-184; M-1 to M-183; M-1 to L-182; M-1 to S-181; M-1 to D-180; M-1 to R-179; M-1 to I-178; M-1 to T-177; M-1 to S-176; M-1 to V-175; M-1 to T-174; M-1 to D-173; M-1 to E-172; M-1 to D-171; M-1 to C-170; M-1 to E-169; M-1 to L-168; M-1 to L-167; M-1 to S-166; M-1 to R-165; M-1 to V-164; M-1 to L-163; M-1 to R-162; M-1 to N-161; M-1 to Y-160; M-1 to Y-159; M-1 to R-158; M-1 to N-157; M-1 to S-156; M-1 to F-155; M-1 to H-154; M-1 to N-153; M-1 to P-152; M-1 to L-151; M-1 to Q-150; M-1 to V-149; M-1 to V-148; M-1 to E-147; M-1 to T-146; M-1 to I-145; M-1 to A-144; M-1 to E-143; M-1 to P-142; M-1 to N-141; M-1 to R-140; M-1 to K-139; M-1 to A-138; M-1 to I-137; M-1 to S-136; M-1 to C-135; M-1 to V-134; M-1 to N-133; M-1 to L-132; M-1 to K-131; M-1 to S-130; M-1 to Y-129; M-1 to C-128; M-1 to E-127; M-1 to E-126; M-1 to Q-125; M-1 to V-124; M-1 to E-123; M-1 to A-122; M-1 to I-121; M-1 to M-120; M-1 to R-119; M-1 to Q-118; M-1 to F-117; M-1 to T-116; M-1 to S-115; M-1 to C-114; M-1 to R-113; M-1 to R-112; M-1 to I-111; M-1 to A-110; M-1 to L-109; M-1 to F-108; M-1 to V-107; M-1 to K-106; M-1 to S-105; M-1 to T-104; M-1 to V-103; M-1 to G-102; M-1 to N-101; M-1 to A-100; M-1 to I-99; M-1 to C-98; M-1 to K-97; M-1 to L-96; M-1 to S-95; M-1 to E-94; M-1 to K-93; M-1 to V-92; M-1 to F-91; M-1 to A-90; M-1 to K-89; M-1 to G-88; M-1 to Q-87; M-1 to T-86; M-1 to D-85; M-1 to F-84; M-1 to K-83; M-1 to A-82; M-1 to A-81; M-1 to S-80; M-1 to Y-79; M-1 to L-78; M-1 to F-77; M-1 to S-76; M-1 to K-75; M-1 to C-74; M-1 to I-73; M-1 to D-72; M-1 to Y-71; M-1 to M-70; M-1 to G-69; M-1 to D-68; M-1 to T-67; M-1 to D-66; M-1 to C-65; M-1 to T-64; M-1 to S-63; M-1 to N-62; M-1 to E-61; M-1 to L-60; M-1 to C-59; M-1 to A-58; M-1 to F-57; M-1 to A-56; M-1 to G-55; M-1 to C-54; M-1 to G-53; M-1 to V-52; M-1 to Q-51; M-1 to L-50; M-1 to A-49; M-1 to S-48; M-1 to N-47; M-1 to L-46; M-1 to C-45; M-1 to R-44; M-1 to V-43; M-1 to V-42; M-1 to E-41; M-1 to A-40; M-1 to S-39; M-1 to N-38; M-1 to Q-37; M-1 to A-36; M-1 to A-35; M-1 to V-34; M-1 to R-33; M-1 to S-32; M-1 to K-31; M-1 to R-30; M-1 to P-29; M-1 to S-28; M-1 to V-27; M-1 to S-26; M-1 to D-25; M-1 to N-24; M-1 to Q-23; M-1 to E-22; M-1 to A-21; M-1 to E-20; M-1 to H-

19; M-1 to T-18; M-1 to A-17; M-1 to S-16; M-1 to A-15; M-1 to S-14; M-1 to I-13; M-1 to V-12; M-1 to L-11; M-1 to V-10; M-1 to L-9; M-1 to L-8; M-1 to V-7; of SEQ ID NO: 2. Polynucleotides encoding these polypeptides are also encompassed by the invention, as are antibodies that bind one or more of these polypeptides. Moreover, fragments and variants of these polypeptides (e.g., fragments as described herein, polypeptides at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to these polypeptides and polypeptides encoded by the polynucleotide which hybridizes, under stringent conditions, to the polynucleotide encoding these polypeptides, or the complement thereof) are encompassed by the invention. Antibodies that bind these fragments and variants of the invention are also encompassed by the invention. Polynucleotides encoding these fragments and variants are also encompassed by the invention.

[0100] Moreover, a signal sequence may be added to these C-terminal constructs. For example, amino acids 1-30 of SEQ ID NO:2, amino acids 2-30 of SEQ ID NO:2, amino acids 3-30 of SEQ ID NO:2, amino acids 4-30 of SEQ ID NO:2, amino acids 5-30 of SEQ ID NO:2, amino acids 6-30 of SEQ ID NO:2, amino acids 7-30 of SEQ ID NO:2, amino acids 8-30 of SEQ ID NO:2, amino acids 9-30 of SEQ ID NO:2, amino acids 10-30 of SEQ ID NO:2, amino acids 11-30 of SEQ ID NO:2, amino acids 12-30 of SEQ ID NO:2, amino acids 13-30 of SEQ ID NO:2, amino acids 14-30 of SEQ ID NO:2, amino acids 15-30 of SEQ ID NO:2, amino acids 16-30 of SEQ ID NO:2, amino acids 17-30 of SEQ ID NO:2, amino acids 18-30 of SEQ ID NO:2, amino acids 19-30 of SEQ ID NO:2, amino acids 20-30 of SEQ ID NO:2, amino acids 21-30 of SEQ ID NO:2, amino acids 22-30 of SEQ ID NO:2, amino acids 23-30 of SEQ ID NO:2, amino acids 24-30 of SEQ ID NO:2, amino acids 25-30 of SEQ ID NO:2, amino acids 26-30 of SEQ ID NO:2, amino acids 27-30 of SEQ ID NO:2, amino acids 28-30 of SEQ ID NO:2, or amino acids 29-30 of SEQ ID NO:2 can be added to the N-terminus of each C-terminal constructs listed above.

[0101] In addition, any of the above listed N- or C-terminal deletions can be combined to produce a N- and C-terminal deleted stanniocalcin polypeptide. The invention also provides polypeptides having one or more amino acids deleted from both the amino and the carboxyl termini, which may be described generally as having residues m-n of SEQ ID NO:2, where n and m are integers as described above. Polynucleotides encoding these polypeptides are also encompassed by the invention, as are antibodies that bind one or more of these polypeptides. Moreover, fragments and variants of these polypeptides (e.g.,

fragments as described herein, polypeptides at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to these polypeptides and polypeptides encoded by the polynucleotide which hybridizes, under stringent conditions, to the polynucleotide encoding these polypeptides, or the complement thereof) are encompassed by the invention. Antibodies that bind these fragments and variants of the invention are also encompassed by the invention. Polynucleotides encoding these fragments and variants are also encompassed by the invention.

[0102] Also included are polypeptides consisting of a portion of the complete stanniocalcin amino acid sequence encoded by the cDNA plasmid contained in ATCC Deposit No. 75652, where this portion excludes any integer of amino acid residues from 1 to about 246 amino acids from the amino terminus of the complete amino acid sequence encoded by the cDNA plasmid contained in ATCC Deposit No. 75652, or any integer of amino acid residues from 1 to 246 amino acids from the carboxy terminus, or any combination of the above amino terminal and carboxy terminal deletions, of the complete amino acid sequence encoded by the cDNA plasmid contained in ATCC Deposit No. 75652. Polynucleotides encoding these polypeptides are also encompassed by the invention, as are antibodies that bind one or more of these polypeptides. Moreover, fragments and variants of these polypeptides (e.g., fragments as described herein, polypeptides at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to these polypeptides and polypeptides encoded by the polynucleotide which hybridizes, under stringent conditions, to the polynucleotide encoding these polypeptides, or the complement thereof) are encompassed by the invention. Antibodies that bind these fragments and variants of the invention are also encompassed by the invention. Polynucleotides encoding these fragments and variants are also encompassed by the invention.

[0103] Among the especially preferred fragments of the invention are fragments characterized by structural or functional attributes of stanniocalcin. Such fragments include amino acid residues that comprise alpha-helix and alpha-helix forming regions ("alpha-regions"), beta-sheet and beta-sheet-forming regions ("beta-regions"), turn and turn-forming regions ("turn-regions"), coil and coil-forming regions ("coil-regions"), hydrophilic regions, hydrophobic regions, alpha amphipathic regions, beta amphipathic regions, surface forming regions, and high antigenic index regions (i.e., containing four or more contiguous amino acids having an antigenic index of greater than or equal to 1.5, as

identified using the default parameters of the Jameson-Wolf program) of complete (i.e., full-length) stanniocalcin (SEQ ID NO:2). Table I, and corresponding Figure 3, show the above listed features of the amino acid sequence presented in SEQ ID NO: 2 and Figures 1A and 1B. The column headings of Table I refer to the following features of the amino acid sequence: "Res": amino acid residue of SEQ ID NO:2 and "Position": position of the corresponding residue within SEQ ID NO:2 and Figures 1A and 1B; I: Alpha, Regions - Garnier-Robson; II: Alpha, Regions - Chou-Fasman; III: Beta, Regions - Garnier-Robson; IV: Beta, Regions - Chou-Fasman; V: Turn, Regions - Garnier-Robson; VI: Turn, Regions - Chou-Fasman; VII: Coil, Regions - Garnier-Robson; VIII: Hydrophilicity Plot - Kyte-Doolittle; IX: Hydrophobicity Plot - Hopp-Woods; X: Alpha, Amphipathic Regions - Eisenberg; XI: Beta, Amphipathic Regions - Eisenberg; XII: Flexible Regions - Karplus-Schulz; XIII: Antigenic Index - Jameson-Wolf; and XIV: Surface Probability Plot - Emini. Certain preferred regions are those set out in Figure 3 and include, but are not limited to, regions of the aforementioned types identified by analysis of the amino acid sequence depicted in Figure 1 (SEQ ID NO:2), such preferred regions include; Garnier-Robson predicted alpha-regions, beta-regions, turn-regions, and coil-regions; Chou-Fasman predicted alpha-regions, beta-regions, turn-regions, and coil-regions; Kyte-Doolittle predicted hydrophilic and hydrophobic regions; Eisenberg alpha and beta amphipathic regions; Emini surface-forming regions; and Jameson-Wolf high antigenic index regions, as predicted using the default parameters of these computer programs. Polynucleotides encoding these polypeptides are also encompassed by the invention, as are antibodies that bind one or more of these polypeptides. Moreover, fragments and variants of these polypeptides (e.g., fragments as described herein, polypeptides at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to these polypeptides and polypeptides encoded by the polynucleotide which hybridizes, under stringent conditions, to the polynucleotide encoding these polypeptides, or the complement thereof) are encompassed by the invention. Antibodies that bind these fragments and variants of the invention are also encompassed by the invention. Polynucleotides encoding these fragments and variants are also encompassed by the invention.

[0104] In additional embodiments, the polynucleotides of the invention encode functional attributes of stanniocalcin. Preferred embodiments of the invention in this regard include fragments that comprise alpha-helix and alpha-helix forming regions

("alpha-regions"), beta-sheet and beta-sheet forming regions ("beta-regions"), turn and turn-forming regions ("turn-regions"), coil and coil-forming regions ("coil-regions"), hydrophilic regions, hydrophobic regions, alpha amphipathic regions, beta amphipathic regions, flexible regions, surface-forming regions and high antigenic index regions of stanniocalcin.

[0105] The data representing the structural or functional attributes of stanniocalcin set forth in Figure 1 and/or Table I, as described above, was generated using the various modules and algorithms of the DNA*STAR set on default parameters. In a preferred embodiment, the data presented in columns VIII, IX, XIII, and XIV of Table I can be used to determine regions of stanniocalcin which exhibit a high degree of potential for antigenicity. Regions of high antigenicity are determined from the data presented in columns VIII, IX, XIII, and/or IV by choosing values which represent regions of the polypeptide which are likely to be exposed on the surface of the polypeptide in an environment in which antigen recognition may occur in the process of initiation of an immune response.

[0106] Certain preferred regions in these regards are set out in Figure 3, but may, as shown in Table I, be represented or identified by using tabular representations of the data presented in Figure 3. The DNA*STAR computer algorithm used to generate Figure 3 (set on the original default parameters) was used to present the data in Figure 3 in a tabular format (See Table I). The tabular format of the data in Figure 3 may be used to easily determine specific boundaries of a preferred region.

[0107] The above-mentioned preferred regions set out in Figure 3 and in Table I include, but are not limited to, regions of the aforementioned types identified by analysis of the amino acid sequence set out in Figure 1. As set out in Figure 3 and in Table I, such preferred regions include Garnier-Robson alpha-regions, beta-regions, turn-regions, and coil-regions, Chou-Fasman alpha-regions, beta-regions, and coil-regions, Kyte-Doolittle hydrophilic regions and hydrophobic regions, Eisenberg alpha- and beta-amphipathic regions, Karplus-Schulz flexible regions, Emini surface-forming regions and Jameson-Wolf regions of high antigenic index.

Table I

Res	Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
Met	1	A	0.23	0.39	.	.	.	-0.10	0.85
Leu	2	A	T	.	0.03	0.34	.	.	.	0.10	0.89

Res	Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
Gln	3	A	T	.	-0.43	0.41	.	.	.	-0.20	0.71
Asn	4	A	T	.	-0.86	0.63	.	.	.	-0.20	0.53
Ser	5	A	T	.	-1.28	0.70	.	.	F	-0.05	0.53
Ala	6	A	.	.	B	.	.	.	-1.53	0.70	.	.	.	-0.60	0.25
Val	7	A	.	.	B	.	.	.	-1.53	0.94	.	.	.	-0.60	0.12
Leu	8	A	.	.	B	.	.	.	-2.39	1.23	.	.	.	-0.60	0.07
Leu	9	A	.	.	B	.	.	.	-3.28	1.49	.	.	.	-0.60	0.05
Val	10	.	.	B	B	.	.	.	-3.28	1.67	.	.	.	-0.60	0.05
Leu	11	A	.	.	B	.	.	.	-3.28	1.41	.	.	.	-0.60	0.08
Val	12	A	.	.	B	.	.	.	-2.72	1.23	.	.	.	-0.60	0.10
Ile	13	A	.	.	B	.	.	.	-2.50	0.93	.	.	.	-0.60	0.18
Ser	14	A	A	-2.00	0.79	.	.	.	-0.60	0.22
Ala	15	A	A	-1.18	0.59	.	.	.	-0.60	0.43
Ser	16	A	A	-0.37	0.44	.	*	.	-0.60	0.83
Ala	17	A	A	-0.10	-0.24	.	*	.	0.45	1.07
Thr	18	A	A	0.79	-0.13	.	*	.	0.45	1.07
His	19	A	A	1.09	-0.63	.	.	.	0.75	1.38
Glu	20	A	A	1.68	-0.61	.	.	.	0.75	2.36
Ala	21	A	A	1.98	-0.71	.	.	F	1.24	2.63
Glu	22	A	A	2.27	-1.20	.	.	F	1.58	3.23
Gln	23	A	A	1.72	-1.31	.	.	F	1.92	2.50
Asn	24	A	T	.	1.46	-0.67	.	.	F	2.66	1.84
Asp	25	T	T	.	1.24	-0.79	.	*	F	3.40	1.42
Ser	26	T	T	.	1.94	-0.36	.	.	F	2.76	1.27
Val	27	T	C	1.99	-0.76	.	.	F	2.86	1.55
Ser	28	T	C	1.69	-1.16	.	.	F	2.86	1.85
Pro	29	T	C	1.80	-0.77	.	*	F	2.86	1.85
Arg	30	T	T	.	0.94	-1.16	.	.	F	3.06	4.88
Lys	31	T	T	.	0.66	-1.16	.	*	F	3.40	2.70
Ser	32	A	A	0.92	-1.04	*	*	F	2.26	1.77
Arg	33	A	A	1.22	-0.97	*	.	F	1.77	0.91
Val	34	A	A	1.43	-0.57	*	*	.	1.28	0.79
Ala	35	A	A	1.02	-0.17	*	*	.	0.64	0.95
Ala	36	A	T	.	0.39	-0.17	.	*	.	0.70	0.65
Gln	37	A	T	.	0.69	0.33	.	*	F	0.25	0.88
Asn	38	A	T	.	-0.28	-0.31	*	*	F	1.00	1.51
Ser	39	A	T	.	-0.28	-0.17	*	.	F	1.00	1.11
Ala	40	A	A	0.42	-0.03	*	.	F	0.45	0.48
Glu	41	A	A	0.34	-0.43	*	.	.	0.30	0.58
Val	42	A	A	-0.47	-0.26	*	.	.	0.30	0.23
Val	43	A	A	-0.47	0.04	*	.	.	-0.30	0.19
Arg	44	A	A	-0.47	-0.06	*	.	.	0.30	0.18
Cys	45	A	T	.	-0.47	0.33	*	.	.	0.10	0.32
Leu	46	A	T	.	-1.28	0.19	*	.	.	0.10	0.43
Asn	47	A	T	.	-0.42	0.23	*	.	.	0.10	0.18
Ser	48	A	T	.	-0.42	0.63	*	*	.	-0.20	0.59
Ala	49	A	-0.88	0.70	*	.	.	-0.40	0.53
Leu	50	A	-0.88	0.44	.	*	.	-0.40	0.33
Gln	51	T	.	.	-0.41	0.61	.	*	.	0.00	0.13
Val	52	T	T	.	-1.00	0.66	.	.	.	0.20	0.13
Gly	53	T	T	.	-1.40	0.66	.	.	.	0.20	0.16
Cys	54	T	T	.	-1.40	0.76	.	.	.	0.20	0.08
Gly	55	T	T	.	-1.26	0.86	.	.	.	0.20	0.11
Ala	56	A	A	-2.07	0.79	.	.	.	-0.60	0.06
Phe	57	A	A	-1.21	1.04	.	.	.	-0.60	0.09
Ala	58	A	A	-0.87	0.47	.	.	.	-0.60	0.16
Cys	59	A	A	-0.50	0.44	.	.	.	-0.60	0.25
Leu	60	A	A	-0.47	0.33	.	.	.	0.01	0.38
Glu	61	.	A	.	.	T	.	.	-0.54	0.03	.	.	F	0.87	0.55
Asn	62	T	T	.	0.16	0.10	.	.	F	1.58	0.55

Res	Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
Ser	63	T	T	.	0.43	-0.47	.	.	F	2.64	1.11
Thr	64	T	T	.	1.10	-0.67	.	.	F	3.10	0.92
Cys	65	T	T	.	1.57	-0.67	.	.	F	2.79	0.96
Asp	66	T	T	.	0.97	-0.64	.	.	F	2.48	0.71
Thr	67	T	T	.	0.72	-0.41	.	*	F	1.87	0.48
Asp	68	T	T	.	1.02	-0.14	.	*	F	1.71	1.42
Gly	69	T	T	.	0.44	-0.71	.	*	.	1.55	1.42
Met	70	A	.	.	B	.	.	.	0.44	-0.03	.	*	.	0.30	0.69
Tyr	71	A	.	.	B	.	.	.	0.49	0.06	*	.	.	-0.30	0.22
Asp	72	A	.	.	B	.	.	.	0.50	0.06	*	.	.	-0.30	0.45
Ile	73	A	.	.	B	.	.	.	-0.20	0.01	*	.	.	-0.30	0.60
Cys	74	A	T	.	-0.67	0.19	*	.	.	0.10	0.33
Lys	75	A	T	.	-0.31	0.11	*	.	.	0.10	0.16
Ser	76	A	T	.	-0.37	0.87	*	.	.	-0.20	0.37
Phe	77	A	T	.	-0.96	0.57	*	.	.	-0.20	0.92
Leu	78	A	A	-0.66	0.50	*	.	.	-0.60	0.47
Tyr	79	A	A	0.06	1.00	*	.	.	-0.60	0.35
Ser	80	A	A	-0.69	0.61	*	*	.	-0.60	0.81
Ala	81	A	A	-0.39	0.61	.	*	.	-0.60	0.85
Ala	82	A	A	0.00	-0.07	.	*	.	0.30	0.91
Lys	83	A	A	0.81	-0.34	.	*	.	0.30	0.98
Phe	84	A	A	0.71	-0.33	.	*	F	0.60	1.67
Asp	85	A	A	1.06	-0.40	.	*	F	0.60	1.64
Thr	86	A	T	.	1.06	-0.90	.	*	F	1.30	1.64
Gln	87	A	T	.	0.94	-0.40	.	*	F	1.00	1.91
Gly	88	A	T	.	0.04	-0.40	*	*	F	0.85	0.99
Lys	89	A	T	.	0.79	0.24	*	*	F	0.25	0.51
Ala	90	A	A	0.79	-0.24	.	*	F	0.45	0.59
Phe	91	A	A	0.80	-0.64	.	*	.	0.75	1.03
Val	92	A	A	-0.01	-0.69	*	*	.	0.60	0.69
Lys	93	A	A	0.38	0.00	*	*	F	-0.15	0.56
Glu	94	A	A	-0.33	-0.50	*	*	F	0.60	1.30
Ser	95	A	A	-0.63	-0.71	*	*	F	0.75	0.94
Leu	96	A	A	-0.52	-0.67	*	*	F	0.75	0.33
Lys	97	A	A	0.33	-0.17	*	*	.	0.30	0.19
Cys	98	A	A	-0.06	0.23	*	*	.	-0.30	0.23
Ile	99	A	T	.	-0.91	0.27	*	.	.	0.10	0.28
Ala	100	A	T	.	-0.92	0.23	*	.	.	0.10	0.10
Asn	101	A	T	.	-0.41	0.71	*	*	.	-0.20	0.28
Gly	102	A	T	.	-0.41	0.53	*	*	F	-0.05	0.53
Val	103	A	.	.	B	.	.	.	-0.60	-0.16	.	.	F	0.60	1.05
Thr	104	A	.	.	B	.	.	.	-0.41	-0.01	.	.	F	0.45	0.48
Ser	105	A	.	.	B	.	.	.	-0.63	0.37	.	.	F	-0.15	0.42
Lys	106	A	.	.	B	.	.	.	-1.22	0.63	.	*	F	-0.45	0.47
Val	107	A	.	.	B	.	.	.	-1.77	0.49	*	*	.	-0.60	0.33
Phe	108	A	.	.	B	.	.	.	-0.80	0.69	*	*	.	-0.60	0.17
Leu	109	A	.	.	B	.	.	.	-0.38	0.30	*	*	.	-0.30	0.17
Ala	110	A	.	.	B	.	.	.	-0.74	0.30	*	*	.	-0.05	0.44
Ile	111	A	.	.	B	.	.	.	-1.09	0.23	*	*	.	0.20	0.28
Arg	112	A	T	.	-0.54	-0.17	*	.	.	1.45	0.45
Arg	113	T	T	.	-0.54	-0.37	.	.	.	2.10	0.64
Cys	114	T	T	.	0.27	-0.09	*	.	F	2.50	0.79
Ser	115	T	T	.	0.97	-0.37	*	.	F	2.25	0.70
Thr	116	.	A	.	.	T	.	.	1.26	-0.37	*	.	F	1.60	0.70
Phe	117	.	A	.	.	T	.	.	0.26	0.24	*	.	.	0.75	1.29
Gln	118	A	A	-0.44	0.36	*	*	.	-0.05	0.67
Arg	119	A	A	0.22	0.47	*	.	.	-0.60	0.47
Met	120	A	A	-0.33	-0.01	*	*	.	0.30	0.94
Ile	121	A	A	-0.02	-0.16	*	.	.	0.30	0.40
Ala	122	A	A	0.68	-0.16	*	*	.	0.30	0.36

Res	Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
Glu	123	A	A	0.68	-0.16	*	*	.	0.30	0.63
Val	124	A	A	-0.10	-0.77	*	*	.	0.75	1.55
Gln	125	A	A	0.26	-0.89	.	.	F	0.75	0.82
Glu	126	A	A	0.84	-0.63	.	*	F	0.75	0.74
Glu	127	A	A	1.48	-0.24	.	*	F	0.60	1.34
Cys	128	A	T	.	0.67	-0.89	.	*	.	1.15	1.55
Tyr	129	A	T	.	1.52	-0.60	.	*	.	1.00	0.74
Ser	130	A	T	.	0.67	-0.20	.	*	.	0.70	0.68
Lys	131	A	T	.	0.00	0.44	.	*	.	-0.20	0.95
Leu	132	A	-0.30	0.44	.	*	.	-0.40	0.32
Asn	133	A	T	.	-0.52	0.07	.	*	.	0.10	0.32
Val	134	A	T	.	-0.87	0.37	.	*	.	0.10	0.11
Cys	135	A	T	.	-0.52	0.87	.	*	.	-0.20	0.14
Ser	136	A	T	.	-0.46	0.19	.	*	.	0.10	0.17
Ile	137	A	0.36	-0.21	*	.	.	0.80	0.46
Ala	138	A	0.14	-0.46	*	.	.	1.25	1.37
Lys	139	T	.	.	1.00	-0.60	*	.	F	2.40	1.58
Arg	140	C	1.08	-0.99	*	.	F	2.50	3.91
Asn	141	T	C	0.49	-1.17	*	.	F	3.00	3.91
Pro	142	T	C	1.07	-0.99	*	.	F	2.70	1.37
Glu	143	A	T	.	1.66	-0.50	*	.	F	1.90	1.01
Ala	144	A	T	.	0.76	-0.50	*	.	.	1.45	1.09
Ile	145	A	.	.	B	.	.	.	-0.21	-0.26	*	.	.	0.60	0.52
Thr	146	A	.	.	B	.	.	.	-0.21	-0.04	*	.	.	0.30	0.22
Glu	147	A	.	.	B	.	.	.	-0.81	0.36	*	.	.	-0.30	0.38
Val	148	A	.	.	B	.	.	.	-1.02	0.54	*	.	.	-0.60	0.45
Val	149	A	.	.	B	.	.	.	-0.43	0.29	*	.	.	-0.30	0.48
Gln	150	A	.	.	B	.	.	.	0.42	0.20	*	.	.	-0.30	0.45
Leu	151	T	C	0.03	0.70	*	.	.	0.00	0.82
Pro	152	T	C	-0.27	0.84	*	.	.	0.00	0.96
Asn	153	T	T	.	0.59	0.59	*	*	.	0.20	0.74
His	154	T	C	1.56	0.59	*	*	.	0.15	1.45
Phe	155	T	.	.	1.31	-0.10	*	*	F	1.45	1.83
Ser	156	T	.	.	1.88	0.23	*	.	F	1.10	1.79
Asn	157	T	T	.	2.09	0.59	*	*	F	1.25	2.06
Arg	158	T	T	.	2.20	0.49	*	*	F	1.50	3.82
Tyr	159	T	T	.	1.42	-0.30	*	*	.	2.50	5.58
Tyr	160	T	T	.	1.27	0.00	*	*	.	1.65	2.86
Asn	161	.	.	.	B	T	.	.	1.68	0.24	*	.	.	1.00	1.09
Arg	162	.	.	.	B	T	.	.	1.38	0.24	*	.	.	0.75	1.36
Leu	163	.	.	B	B	.	.	.	0.46	-0.13	*	*	.	0.70	1.16
Val	164	.	.	B	B	.	.	.	-0.11	-0.20	*	.	.	0.30	0.59
Arg	165	.	A	B	0.13	0.09	*	.	.	-0.30	0.25
Ser	166	A	A	-0.53	0.09	*	*	.	-0.30	0.53
Leu	167	A	A	-0.64	-0.03	*	.	.	0.30	0.38
Leu	168	A	A	0.17	-0.67	*	*	.	0.60	0.32
Glu	169	A	A	1.02	-0.67	*	*	.	0.60	0.42
Cys	170	A	A	0.60	-1.06	*	*	F	0.75	0.85
Asp	171	A	T	.	0.04	-1.26	.	.	F	1.30	1.48
Glu	172	A	T	.	0.56	-1.30	.	.	F	1.15	0.64
Asp	173	A	T	.	1.06	-0.91	.	.	F	1.30	1.59
Thr	174	A	T	.	0.17	-1.00	*	*	F	1.30	1.37
Val	175	A	.	.	B	.	.	.	0.94	-0.31	*	.	F	0.45	0.56
Ser	176	A	.	.	B	.	.	.	0.94	-0.31	.	.	F	0.45	0.65
Thr	177	A	.	.	B	.	.	.	0.64	-0.31	*	*	F	0.45	0.75
Ile	178	A	T	.	-0.17	-0.41	*	*	F	1.00	1.36
Arg	179	A	T	.	-0.46	-0.37	*	.	F	0.85	0.84
Asp	180	A	T	.	0.40	-0.14	.	*	F	0.85	0.57
Ser	181	A	T	.	0.74	-0.63	.	*	.	1.15	1.42
Leu	182	A	A	0.17	-1.31	.	*	.	0.75	1.45

Res	Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
Met	183	A	A	0.71	-0.63	.	*	.	0.60	0.61
Glu	184	A	A	0.39	-0.20	.	*	.	0.30	0.45
Lys	185	A	A	0.39	-0.16	*	*	F	0.45	0.84
Ile	186	A	A	0.09	-0.44	*	*	F	0.60	1.37
Gly	187	T	C	0.31	-0.44	*	.	F	1.05	0.78
Pro	188	A	T	.	0.61	0.06	*	.	F	0.25	0.40
Asn	189	A	T	.	-0.20	0.44	*	.	.	-0.20	0.76
Met	190	A	T	.	-0.94	0.44	*	*	.	-0.20	0.63
Ala	191	A	A	-0.09	0.80	.	.	.	-0.60	0.35
Ser	192	A	A	-0.63	0.87	.	.	.	-0.60	0.30
Leu	193	A	A	-1.23	1.16	.	.	.	-0.60	0.21
Phe	194	A	A	-1.23	1.23	.	.	.	-0.60	0.17
His	195	A	A	-0.94	1.13	.	.	.	-0.60	0.22
Ile	196	A	A	-0.36	1.23	.	.	.	-0.60	0.39
Leu	197	A	A	-0.09	0.54	.	.	.	-0.60	0.75
Gln	198	A	A	0.06	0.26	.	.	.	-0.30	0.75
Thr	199	T	T	.	0.17	0.33	.	.	F	0.65	0.58
Asp	200	T	T	.	0.20	0.14	.	.	F	0.65	0.71
His	201	T	T	.	0.78	-0.14	.	.	.	1.10	0.71
Cys	202	T	T	.	1.56	-0.06	.	*	.	1.10	0.71
Ala	203	T	.	.	1.34	-0.04	*	*	.	1.20	0.57
Gln	204	T	.	.	1.77	0.39	*	*	.	0.90	0.65
Thr	205	C	1.18	-0.11	.	*	F	1.90	2.39
His	206	T	C	1.21	-0.19	.	*	F	2.40	2.39
Pro	207	T	C	1.18	-0.69	.	*	F	3.00	2.30
Arg	208	T	T	.	1.77	-0.30	.	*	F	2.60	1.38
Ala	209	T	T	.	1.88	-0.39	.	*	F	2.64	1.63
Asp	210	T	.	.	2.30	-0.89	.	*	.	2.63	2.07
Phe	211	T	.	.	2.44	-1.31	.	*	.	2.67	2.07
Asn	212	T	T	.	2.34	-1.31	.	*	.	2.91	4.01
Arg	213	T	T	.	2.23	-1.33	.	*	F	3.40	3.46
Arg	214	T	T	.	2.82	-0.93	.	.	F	3.06	6.43
Arg	215	T	T	.	2.61	-1.71	.	.	F	2.72	6.92
Thr	216	T	.	.	3.31	-1.69	.	.	F	2.18	5.47
Asn	217	C	3.36	-1.29	.	.	F	1.64	4.83
Glu	218	.	A	C	2.43	-1.29	.	*	F	1.10	4.93
Pro	219	A	A	2.37	-0.60	.	*	F	0.90	2.82
Gln	220	.	A	.	.	T	.	.	1.40	-1.09	.	*	F	1.30	3.51
Lys	221	A	A	0.90	-0.84	.	*	F	0.90	1.50
Leu	222	A	A	0.09	-0.16	*	*	F	0.45	0.80
Lys	223	A	A	0.20	0.10	*	*	F	-0.15	0.38
Val	224	A	A	0.41	-0.30	*	*	.	0.30	0.37
Leu	225	A	A	-0.40	0.10	*	*	.	-0.30	0.73
Leu	226	A	A	-0.33	0.10	*	*	.	-0.30	0.30
Arg	227	A	A	0.13	0.10	*	*	.	-0.30	0.79
Asn	228	.	A	C	0.09	-0.11	*	*	F	0.65	0.95
Leu	229	.	A	C	0.94	-0.80	*	*	F	1.10	2.00
Arg	230	.	A	C	1.76	-1.49	*	*	F	1.10	1.77
Gly	231	.	A	C	2.27	-1.49	*	*	F	1.40	1.84
Glu	232	.	A	.	.	T	.	.	1.94	-1.50	*	*	F	1.90	2.98
Glu	233	A	A	1.64	-1.76	.	*	F	1.80	2.35
Asp	234	A	A	2.42	-1.37	*	*	F	2.10	3.19
Ser	235	T	C	1.42	-1.30	*	*	F	3.00	2.51
Pro	236	A	T	.	1.81	-0.61	*	.	F	2.50	1.01
Ser	237	A	T	.	1.92	-0.61	*	.	F	2.20	1.21
His	238	A	T	.	1.61	-0.61	*	.	F	2.16	1.77
Ile	239	A	1.31	-0.51	*	.	.	1.77	1.66
Lys	240	A	1.58	-0.56	*	.	F	1.88	1.66
Arg	241	A	1.79	-0.44	*	.	F	1.84	1.66
Thr	242	C	1.79	-0.94	*	.	F	2.60	4.09

Res	Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
Ser	243	C	1.23	-1.24	*	.	F	2.34	2.74
His	244	C	1.73	-0.74	*	.	F	2.08	1.41
Glu	245	A	1.30	-0.31	.	.	.	1.17	1.25
Ser	246	A	0.80	-0.37	.	.	.	0.91	1.19
Ala	247	A	0.72	-0.33	.	.	.	0.65	1.12

[0108] Among highly preferred fragments in this regard are those that comprise regions of stanniocalcin that combine several structural features, such as several of the features set out above.

[0109] Other preferred fragments are biologically active stanniocalcin fragments. Biologically active fragments are those exhibiting activity similar, but not necessarily identical, to an activity of the stanniocalcin polypeptide. The biological activity of the fragments may include an improved desired activity, or a decreased undesirable activity.

Epitope-Bearing Portions

[0110] In another aspect, the invention provides peptides and polypeptides comprising epitope-bearing portions of the polypeptides of the present invention. These epitopes are immunogenic or antigenic epitopes of the polypeptides of the present invention. An “immunogenic epitope” is defined as a part of a protein that elicits an antibody response in vivo when the whole polypeptide of the present invention, or fragment thereof, is the immunogen. On the other hand, a region of a polypeptide to which an antibody can bind is defined as an “antigenic determinant” or “antigenic epitope.” The number of in vivo immunogenic epitopes of a protein generally is less than the number of antigenic epitopes (See, e.g., Geysen *et al.*, *Proc. Natl. Acad. Sci. USA* 81:3998-4002 (1983)). However, antibodies can be made to any antigenic epitope, regardless of whether it is an immunogenic epitope, by using methods such as phage display (See, e.g., Petersen *et al.*, *Mol. Gen. Genet.* 249:425-31 (1995)). Therefore, included in the present invention are both immunogenic epitopes and antigenic epitopes.

[0111] A list of exemplified amino acid sequences comprising immunogenic epitopes are shown in Table 1 above. It is pointed out that Table 1 only lists amino acid residues comprising epitopes predicted to have the highest degree of antigenicity using the algorithm of Jameson and Wolf, *Comp. Appl. Biosci.* 4:181-86 (1988) (said references incorporated by reference in their entireties). The Jameson-Wolf antigenic analysis was performed using the computer program PROTEAN, using default parameters (Version

3.11 for the Power MacIntosh, DNASTAR, Inc., 1228 South Park Street Madison, WI). Table 1 and portions of polypeptides not listed in Table 1 are not considered non-immunogenic. The immunogenic epitopes of Table 1 is an exemplified list, not an exhaustive list, because other immunogenic epitopes are merely not recognized as such by the particular algorithm used. Amino acid residues comprising other immunogenic epitopes may be routinely determined using algorithms similar to the Jameson-Wolf analysis or by in vivo testing for an antigenic response using methods known in the art. See, e.g., Geysen *et al.*, supra; U.S. Patents 4,708,781; 5, 194,392; 4,433,092; and 5,480,971 (said references incorporated by reference in their entireties).

[0112] Antigenic epitope-bearing peptides and polypeptides of the invention preferably contain a sequence of at least seven, more preferably at least nine and most preferably between about 15 to about 30 amino acids contained within the amino acid sequence of a polypeptide of the invention. Non-limiting examples of antigenic polypeptides or peptides that can be used to stanniocalcin -specific antibodies include: a polypeptide comprising amino acid residues in SEQ ID NO:2 from about stanniocalcin. These polypeptide fragments have been determined to bear antigenic epitopes of the stanniocalcin protein by the analysis of the Jameson-Wolf antigenic index, as shown in Figure 3 below.

[0113] It is particularly pointed out that the amino acid sequences of Table 1 comprise immunogenic epitopes. Table 1 lists only the critical residues of immunogenic epitopes determined by the Jameson-Wolf analysis. Thus, additional flanking residues on either the N-terminal, C-terminal, or both N- and C-terminal ends may be added to the sequences of Table 1 to generate an epitope-bearing polypeptide of the present invention. Therefore, the immunogenic epitopes of Table 1 may include additional N-terminal or C-terminal amino acid residues. The additional flanking amino acid residues may be contiguous flanking N-terminal and/or C-terminal sequences from the polypeptides of the present invention, heterologous polypeptide sequences, or may include both contiguous flanking sequences from the polypeptides of the present invention and heterologous polypeptide sequences.

[0114] Polypeptides of the present invention comprising immunogenic or antigenic epitopes are at least 7 amino acids residues in length. "At least" means that a polypeptide of the present invention comprising an immunogenic or antigenic epitope may be 7 amino acid residues in length or any integer between 7 amino acids and the number of amino acid

residues of the full length polypeptides of the invention. [REDACTED]

[REDACTED] However, it is pointed out that each and every integer between 7 and the number of amino acid residues of the full length polypeptide are included in the present invention.

[REDACTED] The immunogenic and antigenic epitope-bearing fragments may be specified by either the number of contiguous amino acid residues, as described above, or further specified by N-terminal and C-terminal positions of these fragments on the amino acid sequence of SEQ ID NO:2. [REDACTED]

[0116] Immunogenic and antigenic epitope-bearing polypeptides of the invention are useful, for example, to make antibodies which specifically bind the polypeptides of the invention, and in immunoassays to detect the polypeptides of the present invention. The antibodies are useful, for example, in affinity purification of the polypeptides of the present invention. The antibodies may also routinely be used in a variety of qualitative or quantitative immunoassays, specifically for the polypeptides of the present invention using methods known in the art. See, e.g., Harlow et al., *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press; 2nd Ed. 1988).

[0117] The epitope-bearing polypeptides of the present invention may be produced by any conventional means for making polypeptides including synthetic and recombinant methods known in the art. For instance, epitope-bearing peptides may be synthesized using known methods of chemical synthesis. For instance, Houghten has described a simple method for the synthesis of large numbers of peptides, such as 10-20 mgs of 248 individual and distinct 13 residue peptides representing single amino acid variants of a segment of the HA1 polypeptide, all of which were prepared and characterized (by ELISA-type binding studies) in less than four weeks (Houghten, R. A., *Proc. Natl. Acad.*

Sci. USA 82:5131-35 (1985)). This "Simultaneous Multiple Peptide Synthesis (SMPS)" process is further described in U.S. Patent No. 4,631,211 to Houghten and coworkers (1986). In this procedure the individual resins for the solid-phase synthesis of various peptides are contained in separate solvent-permeable packets, enabling the optimal use of the many identical repetitive steps involved in solid-phase methods. A completely manual procedure allows 500-1000 or more syntheses to be conducted simultaneously (Houghten *et al.*, *Proc. Natl. Acad. Sci.* 82:5131-35 at 5134 (1985)).

[0118] Epitope-bearing polypeptides of the present invention are used to induce antibodies according to methods well known in the art including, but not limited to, in vivo immunization, in vitro immunization, and phage display methods (See, e.g., Sutcliffe *et al.*, *supra*; Wilson *et al.*, *supra*; Bittle *et al.*, *J. Gen. Virol.* 66:2347-54 (1985)). If in vivo immunization is used, animals may be immunized with free peptide; however, anti-peptide antibody titer may be boosted by coupling of the peptide to a macromolecular carrier, such as keyhole limpet hemacyanin (KLH) or tetanus toxoid. For instance, peptides containing cysteine residues may be coupled to a carrier using a linker such as -maleimidobenzoyl- N-hydroxysuccinimide ester (MBS), while other peptides may be coupled to carriers using a more general linking agent such as glutaraldehyde. Animals such as rabbits, rats and mice are immunized with either free or carrier-coupled peptides, for instance, by intraperitoneal and/or intradermal injection of emulsions containing about 100 μ gs of peptide or carrier protein and Freund's adjuvant. Several booster injections may be needed, for instance, at intervals of about two weeks, to provide a useful titer of anti-peptide antibody which can be detected, for example, by ELISA assay using free peptide adsorbed to a solid surface. The titer of anti-peptide antibodies in serum from an immunized animal may be increased by selection of anti-peptide antibodies, for instance, by adsorption to the peptide on a solid support and elution of the selected antibodies according to methods well known in the art.

[0119] As one of skill in the art will appreciate, and discussed above, the polypeptides of the present invention comprising an immunogenic or antigenic epitope can be fused to heterologous polypeptide sequences. For example, the polypeptides of the present invention may be fused with the constant domain of immunoglobulins (IgA, IgE, IgG, IgM), or portions thereof (CH1, CH2, CH3, any combination thereof including both entire domains and portions thereof) resulting in chimeric polypeptides. By way of another non-

limiting example, polypeptides and/or antibodies of the present invention (including fragments or variants thereof) may be fused with albumin (including but not limited to recombinant human serum albumin or fragments or variants thereof (see, e.g., U.S. Patent No. 5,876,969, issued March 2, 1999, EP Patent 0 413 622, and U.S. Patent No. 5,766,883, issued June 16, 1998, herein incorporated by reference in their entirety)). In a preferred embodiment, polypeptides and/or antibodies of the present invention (including fragments or variants thereof) are fused with the mature form of human serum albumin (i.e., amino acids 1 – 585 of human serum albumin as shown in Figures 1 and 2 of EP Patent 0 322 094) which is herein incorporated by reference in its entirety. In another preferred embodiment, polypeptides and/or antibodies of the present invention (including fragments or variants thereof) are fused with polypeptide fragments comprising, or alternatively consisting of, amino acid residues 1-z of human serum albumin, where z is an integer from 369 to 419, as described in U.S. Patent 5,766,883 herein incorporated by reference in its entirety. Polypeptides and/or antibodies of the present invention (including fragments or variants thereof) may be fused to either the N- or C-terminal end of the heterologous protein (e.g., immunoglobulin Fc polypeptide or human serum albumin polypeptide). Polynucleotides encoding fusion proteins of the invention are also encompassed by the invention.

[0120] Such fusion proteins as those described above may facilitate purification and may increase half-life in vivo. This has been shown, e.g., for chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins (See, e.g., EPA 0,394,827; Traunecker *et al.*, *Nature* 331:84-86 (1988)). Fusion proteins that have a disulfide-linked dimeric structure due to the IgG portion can also be more efficient in binding and neutralizing other molecules than monomeric polypeptides or fragments thereof alone (See, e.g., Fountoulakis *et al.*, *J. Biochem.* 270:3958-64 (1995)). Nucleic acids encoding the above epitopes can also be recombined with a gene of interest as an epitope tag to aid in detection and purification of the expressed polypeptide.

Antibodies

[0121] Further polypeptides of the invention relate to antibodies and T-cell antigen receptors (TCR) which immunospecifically bind a Stanniocalcin polypeptide,

polypeptide fragment, or variant of the invention (e.g., a polypeptide or fragment or variant of the amino acid sequence of SEQ ID NO:2 or a polypeptide encoded by the cDNA contained in the deposited plasmid, and/or an epitope, of the present invention) as determined by immunoassays well known in the art for assaying specific antibody-antigen binding. Antibodies of the invention include, but are not limited to, polyclonal, monoclonal, multispecific, human, humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab') fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies (including, e.g., anti-Id antibodies to antibodies of the invention), intracellularly-made antibodies (i.e., intrabodies), and epitope-binding fragments of any of the above. The term "antibody," as used herein, refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site that immunospecifically binds an antigen. The immunoglobulin molecules of the invention can be of any type (e.g., IgG, IgE, IgM, IgD, IgA and IgY), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. In preferred embodiments, the immunoglobulin molecules of the invention are IgG1. In other preferred embodiments, the immunoglobulin molecules of the invention are IgG4.

[0122] Most preferably the antibodies are human antigen-binding antibody fragments of the present invention and include, but are not limited to, Fab, Fab' and F(ab')₂, Fd, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv) and fragments comprising either a VL or VH domain. Antigen-binding antibody fragments, including single-chain antibodies, may comprise the variable region(s) alone or in combination with the entirety or a portion of the following: hinge region, CH1, CH2, and CH3 domains. Also included in the invention are antigen-binding fragments also comprising any combination of variable region(s) with a hinge region, CH1, CH2, and CH3 domains. The antibodies of the invention may be from any animal origin including birds and mammals. Preferably, the antibodies are human, murine (e.g., mouse and rat), donkey, sheep rabbit, goat, guinea pig, camel, horse, or chicken. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries or from animals transgenic for one or more human immunoglobulin and that do not express endogenous

immunoglobulins, as described *infra* and, for example in, U.S. Patent No. 5,939,598 by Kucherlapati et al.

[0123] The antibodies of the present invention may be monospecific, bispecific, trispecific or of greater multispecificity. Multispecific antibodies may be specific for different epitopes of a polypeptide of the present invention or may be specific for both a polypeptide of the present invention as well as for a heterologous epitope, such as a heterologous polypeptide or solid support material. See, e.g., PCT publications WO 93/17715; WO 92/08802; WO 91/00360; WO 92/05793; Tutt, et al., J. Immunol. 147:60-69 (1991); U.S. Patent Nos. 4,474,893; 4,714,681; 4,925,648; 5,573,920; 5,601,819; Kostelny et al., J. Immunol. 148:1547-1553 (1992).

[0124] Antibodies of the present invention may be described or specified in terms of the epitope(s) or portion(s) of a polypeptide of the present invention which they recognize or specifically bind. The epitope(s) or polypeptide portion(s) may be specified as described herein, e.g., by N-terminal and C-terminal positions, or by size in contiguous amino acid residues. Antibodies which specifically bind any epitope or polypeptide of the present invention may also be excluded. Therefore, the present invention includes antibodies that specifically bind polypeptides of the present invention, and allows for the exclusion of the same.

[0125] Antibodies of the present invention may also be described or specified in terms of their cross-reactivity. Antibodies that do not bind any other analog, ortholog, or homolog of a polypeptide of the present invention are included. Antibodies that bind polypeptides with at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 65%, at least 60%, at least 55%, and at least 50% identity (as calculated using methods known in the art and described herein) to a polypeptide of the present invention are also included in the present invention. In specific embodiments, antibodies of the present invention cross-react with murine, rat and/or rabbit homologs of human proteins and the corresponding epitopes thereof. Antibodies that do not bind polypeptides with less than 95%, less than 90%, less than 85%, less than 80%, less than 75%, less than 70%, less than 65%, less than 60%, less than 55%, and less than 50% identity (as calculated using methods known in the art and described herein) to a polypeptide of the present invention are also included in the present invention. In a specific embodiment, the above-described cross-reactivity is with respect to any single specific antigenic or

immunogenic polypeptide, or combination(s) of 2, 3, 4, 5, or more of the specific antigenic and/or immunogenic polypeptides disclosed herein. Further included in the present invention are antibodies which bind polypeptides encoded by polynucleotides which hybridize to a polynucleotide of the present invention under stringent hybridization conditions (as described herein). Antibodies of the present invention may also be described or specified in terms of their binding affinity to a polypeptide of the invention. Preferred binding affinities include those with a dissociation constant or K_d less than 5×10^{-2} M, 10^{-2} M, 5×10^{-3} M, 10^{-3} M, 5×10^{-4} M, 10^{-4} M, 5×10^{-5} M, 10^{-5} M, 5×10^{-6} M, 10^{-6} M, 5×10^{-7} M, 10^{-7} M, 5×10^{-8} M, 10^{-8} M, 5×10^{-9} M, 10^{-9} M, 5×10^{-10} M, 10^{-10} M, 5×10^{-11} M, 10^{-11} M, 5×10^{-12} M, 10^{-12} M, 5×10^{-13} M, 10^{-13} M, 5×10^{-14} M, 10^{-14} M, 5×10^{-15} M, or 10^{-15} M.

[0126] The invention also provides antibodies that competitively inhibit binding of an antibody to an epitope of the invention as determined by any method known in the art for determining competitive binding, for example, the immunoassays described herein. In preferred embodiments, the antibody competitively inhibits binding to the epitope by at least 95%, at least 90%, at least 85 %, at least 80%, at least 75%, at least 70%, at least 60%, or at least 50%.

[0127] Antibodies of the present invention may act as agonists or antagonists of the polypeptides of the present invention. For example, the present invention includes antibodies which disrupt the receptor/ligand interactions with the polypeptides of the invention either partially or fully. Preferably, antibodies of the present invention bind an antigenic epitope disclosed herein, or a portion thereof. The invention features both receptor-specific antibodies and ligand-specific antibodies. The invention also features receptor-specific antibodies which do not prevent ligand binding but prevent receptor activation. Receptor activation (i.e., signaling) may be determined by techniques described herein or otherwise known in the art. For example, receptor activation can be determined by detecting the phosphorylation (e.g., tyrosine or serine/threonine) of the receptor or its substrate by immunoprecipitation followed by western blot analysis (for example, as described *supra*). In specific embodiments, antibodies are provided that inhibit ligand activity or receptor activity by at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 60%, or at least 50% of the activity in absence of the antibody.

[0128] The invention also features receptor-specific antibodies which both prevent ligand binding and receptor activation as well as antibodies that recognize the receptor-ligand complex, and, preferably, do not specifically recognize the unbound receptor or the unbound ligand. Likewise, included in the invention are neutralizing antibodies which bind the ligand and prevent binding of the ligand to the receptor, as well as antibodies which bind the ligand, thereby preventing receptor activation, but do not prevent the ligand from binding the receptor. Further included in the invention are antibodies which activate the receptor. These antibodies may act as receptor agonists, i.e., potentiate or activate either all or a subset of the biological activities of the ligand-mediated receptor activation, for example, by inducing dimerization of the receptor. The antibodies may be specified as agonists, antagonists or inverse agonists for biological activities comprising the specific biological activities of the peptides of the invention disclosed herein. The above antibody agonists can be made using methods known in the art. See, e.g., PCT publication WO 96/40281; U.S. Patent No. 5,811,097; Deng et al., Blood 92(6):1981-1988 (1998); Chen et al., Cancer Res. 58(16):3668-3678 (1998); Harrop et al., J. Immunol. 161(4):1786-1794 (1998); Zhu et al., Cancer Res. 58(15):3209-3214 (1998); Yoon et al., J. Immunol. 160(7):3170-3179 (1998); Prat et al., J. Cell. Sci. 111(Pt2):237-247 (1998); Pitard et al., J. Immunol. Methods 205(2):177-190 (1997); Liautard et al., Cytokine 9(4):233-241 (1997); Carlson et al., J. Biol. Chem. 272(17):11295-11301 (1997); Taryman et al., Neuron 14(4):755-762 (1995); Muller et al., Structure 6(9):1153-1167 (1998); Bartunek et al., Cytokine 8(1):14-20 (1996) (which are all incorporated by reference herein in their entireties).

[0129] Antibodies of the present invention may be used, for example, to purify, detect, and target the polypeptides of the present invention, including both *in vitro* and *in vivo* diagnostic and therapeutic methods. For example, the antibodies have utility in immunoassays for qualitatively and quantitatively measuring levels of the polypeptides of the present invention in biological samples. See, e.g., Harlow et al., Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); incorporated by reference herein in its entirety.

[0130] As discussed in more detail below, the antibodies of the present invention may be used either alone or in combination with other compositions. The antibodies may further be recombinantly fused to a heterologous polypeptide at the N- or C-terminus or

chemically conjugated (including covalent and non-covalent conjugations) to polypeptides or other compositions. For example, antibodies of the present invention may be recombinantly fused or conjugated to molecules useful as labels in detection assays and effector molecules such as heterologous polypeptides, drugs, radionucleotides, or toxins. See, e.g., PCT publications WO 92/08495; WO 91/14438; WO 89/12624; U.S. Patent No. 5,314,995; and EP 396,387; the disclosures of which are incorporated herein by reference in their entireties.

[0131] The antibodies of the invention include derivatives that are modified, i.e., by the covalent attachment of any type of molecule to the antibody such that covalent attachment does not prevent the antibody from generating an anti-idiotypic response. For example, but not by way of limitation, the antibody derivatives include antibodies that have been modified, e.g., by glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, etc. Additionally, the derivative may contain one or more non-classical amino acids.

[0132] The antibodies of the present invention may be generated by any suitable method known in the art. Polyclonal antibodies to an antigen-of-interest can be produced by various procedures well known in the art. For example, a polypeptide of the invention can be administered to various host animals including, but not limited to, rabbits, mice, rats, etc. to induce the production of sera containing polyclonal antibodies specific for the antigen. Various adjuvants may be used to increase the immunological response, depending on the host species, and include but are not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum. Such adjuvants are also well known in the art.

[0133] Monoclonal antibodies can be prepared using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof. For example, monoclonal antibodies can be

produced using hybridoma techniques including those known in the art and taught, for example, in Harlow et al., *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling, et al., in: *Monoclonal Antibodies and T-Cell Hybridomas* 563-681 (Elsevier, N.Y., 1981) (said references incorporated by reference in their entireties). The term “monoclonal antibody” as used herein is not limited to antibodies produced through hybridoma technology. The term “monoclonal antibody” refers to an antibody that is derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, and not the method by which it is produced.

[0134] Methods for producing and screening for specific antibodies using hybridoma technology are routine and well known in the art and are discussed in detail in the Examples. In a non-limiting example, mice can be immunized with a polypeptide of the invention or a cell expressing such peptide. Once an immune response is detected, e.g., antibodies specific for the antigen are detected in the mouse serum, the mouse spleen is harvested and splenocytes isolated. The splenocytes are then fused by well known techniques to any suitable myeloma cells, for example cells from cell line SP20 available from the ATCC. Hybridomas are selected and cloned by limited dilution. The hybridoma clones are then assayed by methods known in the art for cells that secrete antibodies capable of binding a polypeptide of the invention. Ascites fluid, which generally contains high levels of antibodies, can be generated by immunizing mice with positive hybridoma clones.

[0135] Accordingly, the present invention provides methods of generating monoclonal antibodies as well as antibodies produced by the method comprising culturing a hybridoma cell secreting an antibody of the invention wherein, preferably, the hybridoma is generated by fusing splenocytes isolated from a mouse immunized with an antigen of the invention with myeloma cells and then screening the hybridomas resulting from the fusion for hybridoma clones that secrete an antibody able to bind a polypeptide of the invention.

[0136] Another well known method for producing both polyclonal and monoclonal human B cell lines is transformation using Epstein Barr Virus (EBV). Protocols for generating EBV-transformed B cell lines are commonly known in the art, such as, for example, the protocol outlined in Chapter 7.22 of *Current Protocols in Immunology*, Coligan et al., Eds., 1994, John Wiley & Sons, NY, which is hereby incorporated in its entirety by reference herein. The source of B cells for transformation is commonly human

peripheral blood, but B cells for transformation may also be derived from other sources including, but not limited to, lymph nodes, tonsil, spleen, tumor tissue, and infected tissues. Tissues are generally made into single cell suspensions prior to EBV transformation. Additionally, steps may be taken to either physically remove or inactivate T cells (e.g., by treatment with cyclosporin A) in B cell-containing samples, because T cells from individuals seropositive for anti-EBV antibodies can suppress B cell immortalization by EBV.

[0137] In general, the sample containing human B cells is inoculated with EBV, and cultured for 3-4 weeks. A typical source of EBV is the culture supernatant of the B95-8 cell line (ATCC #VR-1492). Physical signs of EBV transformation can generally be seen towards the end of the 3-4 week culture period. By phase-contrast microscopy, transformed cells may appear large, clear, hairy and tend to aggregate in tight clusters of cells. Initially, EBV lines are generally polyclonal. However, over prolonged periods of cell cultures, EBV lines may become monoclonal or polyclonal as a result of the selective outgrowth of particular B cell clones. Alternatively, polyclonal EBV transformed lines may be subcloned (e.g., by limiting dilution culture) or fused with a suitable fusion partner and plated at limiting dilution to obtain monoclonal B cell lines. Suitable fusion partners for EBV transformed cell lines include mouse myeloma cell lines (e.g., SP2/0, X63-Ag8.653), heteromyeloma cell lines (human x mouse; e.g., SPAM-8, SBC-H20, and CB-F7), and human cell lines (e.g., GM 1500, SKO-007, RPMI 8226, and KR-4). Thus, the present invention also provides a method of generating polyclonal or monoclonal human antibodies against polypeptides of the invention or fragments thereof, comprising EBV-transformation of human B cells.

[0138] Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, Fab and F(ab')₂ fragments of the invention may be produced by proteolytic cleavage of immunoglobulin molecules, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')₂ fragments). F(ab')₂ fragments contain the variable region, the light chain constant region and the CH1 domain of the heavy chain.

[0139] For example, the antibodies of the present invention can also be generated using various phage display methods known in the art. In phage display methods, functional antibody domains are displayed on the surface of phage particles which carry the

polynucleotide sequences encoding them. In a particular embodiment, such phage can be utilized to display antigen binding domains expressed from a repertoire or combinatorial antibody library (e.g., human or murine). Phage expressing an antigen binding domain that binds the antigen of interest can be selected or identified with antigen, e.g., using labeled antigen or antigen bound or captured to a solid surface or bead. Phage used in these methods are typically filamentous phage including fd and M13 binding domains expressed from phage with Fab, Fv or disulfide stabilized Fv antibody domains recombinantly fused to either the phage gene III or gene VIII protein. Examples of phage display methods that can be used to make the antibodies of the present invention include those disclosed in Brinkman et al., J. Immunol. Methods 182:41-50 (1995); Ames et al., J. Immunol. Methods 184:177-186 (1995); Kettleborough et al., Eur. J. Immunol. 24:952-958 (1994); Persic et al., Gene 187 9-18 (1997); Burton et al., Advances in Immunology 57:191-280 (1994); PCT application No. PCT/GB91/01134; PCT publications WO 90/02809; WO 91/10737; WO 92/01047; WO 92/18619; WO 93/11236; WO 95/15982; WO 95/20401; and U.S. Patent Nos. 5,698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821,047; 5,571,698; 5,427,908; 5,516,637; 5,780,225; 5,658,727; 5,733,743 and 5,969,108; each of which is incorporated herein by reference in its entirety.

[0140] As described in the above references, after phage selection, the antibody coding regions from the phage can be isolated and used to generate whole antibodies, including human antibodies, or any other desired antigen binding fragment, and expressed in any desired host, including mammalian cells, insect cells, plant cells, yeast, and bacteria, e.g., as described in detail below. For example, techniques to recombinantly produce Fab, Fab' and F(ab')₂ fragments can also be employed using methods known in the art such as those disclosed in PCT publication WO 92/22324; Mullinax et al., BioTechniques 12(6):864-869 (1992); and Sawai et al., AJRI 34:26-34 (1995); and Better et al., Science 240:1041-1043 (1988) (said references incorporated by reference in their entireties).

[0141] Examples of techniques which can be used to produce single-chain Fvs and antibodies include those described in U.S. Patents 4,946,778 and 5,258,498; Huston et al., Methods in Enzymology 203:46-88 (1991); Shu et al., PNAS 90:7995-7999 (1993); and Skerra et al., Science 240:1038-1040 (1988). For some uses, including *in vivo* use of antibodies in humans and *in vitro* detection assays, it may be preferable to use chimeric, humanized, or human antibodies. A chimeric antibody is a molecule in which different

portions of the antibody are derived from different animal species, such as antibodies having a variable region derived from a murine monoclonal antibody and a human immunoglobulin constant region. Methods for producing chimeric antibodies are known in the art. See e.g., Morrison, Science 229:1202 (1985); Oi et al., BioTechniques 4:214 (1986); Gillies et al., (1989) J. Immunol. Methods 125:191-202; U.S. Patent Nos. 5,807,715; 4,816,567; and 4,816,397, which are incorporated herein by reference in their entirety. Humanized antibodies are antibody molecules from non-human species antibody that binds the desired antigen having one or more complementarity determining regions (CDRs) from the non-human species and a framework region from a human immunoglobulin molecule. Often, framework residues in the human framework regions will be substituted with the corresponding residue from the CDR donor antibody to alter, preferably improve, antigen binding. These framework substitutions are identified by methods well known in the art, e.g., by modeling of the interactions of the CDR and framework residues to identify framework residues important for antigen binding and sequence comparison to identify unusual framework residues at particular positions. (See, e.g., Queen et al., U.S. Patent No. 5,585,089; Riechmann et al., Nature 332:323 (1988), which are incorporated herein by reference in their entirety.) Antibodies can be humanized using a variety of techniques known in the art including, for example, CDR-grafting (EP 239,400; PCT publication WO 91/09967; U.S. Patent Nos. 5,225,539; 5,530,101; and 5,585,089), veneering or resurfacing (EP 592,106; EP 519,596; Padlan, Molecular Immunology 28(4/5):489-498 (1991); Studnicka et al., Protein Engineering 7(6):805-814 (1994); Roguska. et al., PNAS 91:969-973 (1994)), and chain shuffling (U.S. Patent No. 5,565,332).

[0142] Completely human antibodies are particularly desirable for therapeutic treatment of human patients. Human antibodies can be made by a variety of methods known in the art including phage display methods described above using antibody libraries derived from human immunoglobulin sequences. See also, U.S. Patent Nos. 4,444,887 and 4,716,111; and PCT publications WO 98/46645, WO 98/50433, WO 98/24893, WO 98/16654, WO 96/34096, WO 96/33735, and WO 91/10741; each of which is incorporated herein by reference in its entirety.

[0143] Human antibodies can also be produced using transgenic mice which are incapable of expressing functional endogenous immunoglobulins, but which can express

human immunoglobulin genes. For example, the human heavy and light chain immunoglobulin gene complexes may be introduced randomly or by homologous recombination into mouse embryonic stem cells. Alternatively, the human variable region, constant region, and diversity region may be introduced into mouse embryonic stem cells in addition to the human heavy and light chain genes. The mouse heavy and light chain immunoglobulin genes may be rendered non-functional separately or simultaneously with the introduction of human immunoglobulin loci by homologous recombination. In particular, homozygous deletion of the JH region prevents endogenous antibody production. The modified embryonic stem cells are expanded and microinjected into blastocysts to produce chimeric mice. The chimeric mice are then bred to produce homozygous offspring which express human antibodies. The transgenic mice are immunized in the normal fashion with a selected antigen, e.g., all or a portion of a polypeptide of the invention. Monoclonal antibodies directed against the antigen can be obtained from the immunized, transgenic mice using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this technology for producing human antibodies, see Lonberg and Huszar, *Int. Rev. Immunol.* 13:65-93 (1995). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, see, e.g., PCT publications WO 98/24893; WO 92/01047; WO 96/34096; WO 96/33735; European Patent No. 0 598 877; U.S. Patent Nos. 5,413,923; 5,625,126; 5,633,425; 5,569,825; 5,661,016; 5,545,806; 5,814,318; 5,885,793; 5,916,771; 5,939,598; 6,075,181 and 6,114,598, which are incorporated by reference herein in their entirety. In addition, companies such as Abgenix, Inc. (Freemont, CA) and Genpharm (San Jose, CA) can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above.

[0144] Completely human antibodies which recognize a selected epitope can be generated using a technique referred to as “guided selection.” In this approach a selected non-human monoclonal antibody, e.g., a mouse antibody, is used to guide the selection of

a completely human antibody recognizing the same epitope. (Jespers et al., *Bio/technology* 12:899-903 (1988)).

[0145] Further, antibodies to the polypeptides of the invention can, in turn, be utilized to generate anti-idiotypic antibodies that “mimic” polypeptides of the invention using techniques well known to those skilled in the art. (See, e.g., Greenspan & Bona, *FASEB J.* 7(5):437-444; (1989) and Nissinoff, J. *Immunol.* 147(8):2429-2438 (1991)). For example, antibodies which bind to and competitively inhibit polypeptide multimerization and/or binding of a polypeptide of the invention to a ligand can be used to generate anti-idiotypes that “mimic” the polypeptide multimerization and/or binding domain and, as a consequence, bind to and neutralize polypeptide and/or its ligand. Such neutralizing anti-idiotypes or Fab fragments of such anti-idiotypes can be used in therapeutic regimens to neutralize polypeptide ligand/receptor. For example, such anti-idiotypic antibodies can be used to bind a polypeptide of the invention and/or to bind its ligand(s)/receptor(s), and thereby block its biological activity. Alternatively, antibodies which bind to and enhance polypeptide multimerization and/or binding, and/or receptor/ligand multimerization, binding and/or signaling can be used to generate anti-idiotypes that function as agonists of a polypeptide of the invention and/or its ligand/receptor. Such agonistic anti-idiotypes or Fab fragments of such anti-idiotypes can be used in therapeutic regimens as agonists of the polypeptides of the invention or its ligand(s)/receptor(s). For example, such anti-idiotypic antibodies can be used to bind a polypeptide of the invention and/or to bind its ligand(s)/receptor(s), and thereby promote or enhance its biological activity.

[0146] Intrabodies of the invention can be produced using methods known in the art, such as those disclosed and reviewed in Chen *et al.*, *Hum. Gene Ther.* 5:595-601 (1994); Marasco, W.A., *Gene Ther.* 4:11-15 (1997); Rondon and Marasco, *Annu. Rev. Microbiol.* 51:257-283 (1997); Proba *et al.*, *J. Mol. Biol.* 275:245-253 (1998); Cohen *et al.*, *Oncogene* 17:2445-2456 (1998); Ohage and Steipe, *J. Mol. Biol.* 291:1119-1128 (1999); Ohage *et al.*, *J. Mol. Biol.* 291:1129-1134 (1999); Wirtz and Steipe, *Protein Sci.* 8:2245-2250 (1999); Zhu *et al.*, *J. Immunol. Methods* 231:207-222 (1999); and references cited therein.

Polynucleotides Encoding Antibodies

[0147] The invention further provides polynucleotides comprising a nucleotide sequence encoding an antibody of the invention and fragments thereof. The invention also

encompasses polynucleotides that hybridize under stringent or alternatively, under lower stringency hybridization conditions, e.g., as defined herein, to polynucleotides that encode an antibody, preferably, that specifically binds to a polypeptide of the invention, preferably, an antibody that binds to a polypeptide having the amino acid sequence of SEQ ID NO:2 and/or to a polypeptide encoded by the cDNA contained in the deposited plasmid.

[0148] The polynucleotides may be obtained, and the nucleotide sequence of the polynucleotides determined, by any method known in the art. For example, if the nucleotide sequence of the antibody is known, a polynucleotide encoding the antibody may be assembled from chemically synthesized oligonucleotides (e.g., as described in Kutmeier et al., *BioTechniques* 17:242 (1994)), which, briefly, involves the synthesis of overlapping oligonucleotides containing portions of the sequence encoding the antibody, annealing and ligating of those oligonucleotides, and then amplification of the ligated oligonucleotides by PCR.

[0149] Alternatively, a polynucleotide encoding an antibody may be generated from nucleic acid from a suitable source. If a clone containing a nucleic acid encoding a particular antibody is not available, but the sequence of the antibody molecule is known, a nucleic acid encoding the immunoglobulin may be chemically synthesized or obtained from a suitable source (e.g., an antibody cDNA library, or a cDNA library generated from, or nucleic acid, preferably poly A+ RNA, isolated from, any tissue or cells expressing the antibody, such as hybridoma cells selected to express an antibody of the invention) by PCR amplification using synthetic primers hybridizable to the 3' and 5' ends of the sequence or by cloning using an oligonucleotide probe specific for the particular gene sequence to identify, e.g., a cDNA clone from a cDNA library that encodes the antibody. Amplified nucleic acids generated by PCR may then be cloned into replicable cloning vectors using any method well known in the art.

[0150] Once the nucleotide sequence and corresponding amino acid sequence of the antibody is determined, the nucleotide sequence of the antibody may be manipulated using methods well known in the art for the manipulation of nucleotide sequences, e.g., recombinant DNA techniques, site directed mutagenesis, PCR, etc. (see, for example, the techniques described in Sambrook et al., 1990, *Molecular Cloning, A Laboratory Manual*, 2d Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY and Ausubel et al., eds.,

1998, Current Protocols in Molecular Biology, John Wiley & Sons, NY, which are both incorporated by reference herein in their entireties), to generate antibodies having a different amino acid sequence, for example to create amino acid substitutions, deletions, and/or insertions.

[0151] In a specific embodiment, the amino acid sequence of the heavy and/or light chain variable domains may be inspected to identify the sequences of the complementarity determining regions (CDRs) by methods that are well known in the art, e.g., by comparison to known amino acid sequences of other heavy and light chain variable regions to determine the regions of sequence hypervariability. Using routine recombinant DNA techniques, one or more of the CDRs may be inserted within framework regions, e.g., into human framework regions to humanize a non-human antibody, as described *supra*. The framework regions may be naturally occurring or consensus framework regions, and preferably human framework regions (see, e.g., Chothia et al., J. Mol. Biol. 278: 457-479 (1998) for a listing of human framework regions). Preferably, the polynucleotide generated by the combination of the framework regions and CDRs encodes an antibody that specifically binds a polypeptide of the invention. Preferably, as discussed *supra*, one or more amino acid substitutions may be made within the framework regions, and, preferably, the amino acid substitutions improve binding of the antibody to its antigen. Additionally, such methods may be used to make amino acid substitutions or deletions of one or more variable region cysteine residues participating in an intrachain disulfide bond to generate antibody molecules lacking one or more intrachain disulfide bonds. Other alterations to the polynucleotide are encompassed by the present invention and within the skill of the art.

[0152] In addition, techniques developed for the production of “chimeric antibodies” (Morrison et al., Proc. Natl. Acad. Sci. 81:851-855 (1984); Neuberger et al., Nature 312:604-608 (1984); Takeda et al., Nature 314:452-454 (1985)) by splicing genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. As described *supra*, a chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region, e.g., humanized antibodies.

[0153] Alternatively, techniques described for the production of single chain antibodies (U.S. Patent No. 4,946,778; Bird, Science 242:423- 42 (1988); Huston et al., Proc. Natl. Acad. Sci. USA 85:5879-5883 (1988); and Ward et al., Nature 334:544-54 (1989)) can be adapted to produce single chain antibodies. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide. Techniques for the assembly of functional Fv fragments in *E. coli* may also be used (Skerra et al., Science 242:1038- 1041 (1988)).

Methods of Producing Antibodies

[0154] The antibodies of the invention can be produced by any method known in the art for the synthesis of antibodies, in particular, by chemical synthesis or preferably, by recombinant expression techniques. Methods of producing antibodies include, but are not limited to, hybridoma technology, EBV transformation, and other methods discussed herein as well as through the use recombinant DNA technology, as discussed below.

[0155] Recombinant expression of an antibody of the invention, or fragment, derivative or analog thereof, (e.g., a heavy or light chain of an antibody of the invention or a single chain antibody of the invention), requires construction of an expression vector containing a polynucleotide that encodes the antibody. Once a polynucleotide encoding an antibody molecule or a heavy or light chain of an antibody, or portion thereof (preferably containing the heavy or light chain variable domain), of the invention has been obtained, the vector for the production of the antibody molecule may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing a protein by expressing a polynucleotide containing an antibody encoding nucleotide sequence are described herein. Methods which are well known to those skilled in the art can be used to construct expression vectors containing antibody coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. The invention, thus, provides replicable vectors comprising a nucleotide sequence encoding an antibody molecule of the invention, or a heavy or light chain thereof, or a heavy or light chain variable domain, operably linked to a promoter. Such vectors may include the nucleotide sequence encoding the constant region of the antibody molecule (see, e.g., PCT Publication WO 86/05807; PCT Publication WO 89/01036; and

U.S. Patent No. 5,122,464) and the variable domain of the antibody may be cloned into such a vector for expression of the entire heavy or light chain.

[0156] The expression vector is transferred to a host cell by conventional techniques and the transfected cells are then cultured by conventional techniques to produce an antibody of the invention. Thus, the invention includes host cells containing a polynucleotide encoding an antibody of the invention, or a heavy or light chain thereof, or a single chain antibody of the invention, operably linked to a heterologous promoter. In preferred embodiments for the expression of double-chained antibodies, vectors encoding both the heavy and light chains may be co-expressed in the host cell for expression of the entire immunoglobulin molecule, as detailed below.

[0157] A variety of host-expression vector systems may be utilized to express the antibody molecules of the invention. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express an antibody molecule of the invention in situ. These include but are not limited to microorganisms such as bacteria (e.g., *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing antibody coding sequences; yeast (e.g., *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing antibody coding sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing antibody coding sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing antibody coding sequences; or mammalian cell systems (e.g., COS, CHO, BHK, 293, 3T3 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter). Preferably, bacterial cells such as *Escherichia coli*, and more preferably, eukaryotic cells, especially for the expression of whole recombinant antibody molecule, are used for the expression of a recombinant antibody molecule. For example, mammalian cells such as Chinese hamster ovary cells (CHO), in conjunction with a vector such as the major intermediate early gene promoter element from human cytomegalovirus is an

effective expression system for antibodies (Foecking et al., Gene 45:101 (1986); Cockett et al., Bio/Technology 8:2 (1990)).

[0158] In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the antibody molecule being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of an antibody molecule, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther et al., EMBO J. 2:1791 (1983)), in which the antibody coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, Nucleic Acids Res. 13:3101-3109 (1985); Van Heeke & Schuster, J. Biol. Chem. 24:5503-5509 (1989)); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to matrix glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

[0159] In an insect system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The antibody coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter).

[0160] In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the antibody coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the antibody molecule in infected hosts. (e.g., see Logan & Shenk, Proc. Natl. Acad. Sci. USA 81:355-359 (1984)). Specific initiation signals may also be required for efficient translation of inserted antibody

coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al., *Methods in Enzymol.* 153:51-544 (1987)).

[0161] In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERY, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and in particular, breast cancer cell lines such as, for example, BT483, Hs578T, HTB2, BT20 and T47D, and normal mammary gland cell line such as, for example, CRL7030 and Hs578Bst.

[0162] For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the antibody molecule may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the antibody molecule. Such engineered cell lines may be particularly useful in

screening and evaluation of compounds that interact directly or indirectly with the antibody molecule.

[0163] A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler et al., *Cell* 11:223 (1977)), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, *Proc. Natl. Acad. Sci. USA* 48:202 (1992)), and adenine phosphoribosyltransferase (Lowy et al., *Cell* 22:817 (1980)) genes can be employed in tk-, hgp^rt- or ap^rt- cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler et al., *Natl. Acad. Sci. USA* 77:357 (1980); O'Hare et al., *Proc. Natl. Acad. Sci. USA* 78:1527 (1981)); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, *Proc. Natl. Acad. Sci. USA* 78:2072 (1981)); neo, which confers resistance to the aminoglycoside G-418 *Clinical Pharmacy* 12:488-505; Wu and Wu, *Biotherapy* 3:87-95 (1991); Tolstoshev, *Ann. Rev. Pharmacol. Toxicol.* 32:573-596 (1993); Mulligan, *Science* 260:926-932 (1993); and Morgan and Anderson, *Ann. Rev. Biochem.* 62:191-217 (1993); TIB TECH 11(5):155-215 (1993)); and hyg^r, which confers resistance to hygromycin (Santerre et al., *Gene* 30:147 (1984)). Methods commonly known in the art of recombinant DNA technology may be routinely applied to select the desired recombinant clone, and such methods are described, for example, in Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, NY (1993); Kriegler, *Gene Transfer and Expression, A Laboratory Manual*, Stockton Press, NY (1990); and in Chapters 12 and 13, Dracopoli et al. (eds), *Current Protocols in Human Genetics*, John Wiley & Sons, NY (1994); Colberre-Garapin et al., *J. Mol. Biol.* 150:1 (1981), which are incorporated by reference herein in their entireties.

[0164] The expression levels of an antibody molecule can be increased by vector amplification (for a review, see Bebbington and Hentschel, *The use of vectors based on gene amplification for the expression of cloned genes in mammalian cells in DNA cloning*, Vol.3. (Academic Press, New York, 1987)). When a marker in the vector system expressing antibody is amplifiable, increase in the level of inhibitor present in culture of host cell will increase the number of copies of the marker gene. Since the amplified region is associated with the antibody gene, production of the antibody will also increase (Crouse et al., *Mol. Cell. Biol.* 3:257 (1983)).

[0165] Vectors which use glutamine synthase (GS) or DHFR as the selectable markers can be amplified in the presence of the drugs methionine sulfoximine or methotrexate, respectively. An advantage of glutamine synthase based vectors are the availability of cell lines (e.g., the murine myeloma cell line, NS0) which are glutamine synthase negative. Glutamine synthase expression systems can also function in glutamine synthase expressing cells (e.g. Chinese Hamster Ovary (CHO) cells) by providing additional inhibitor to prevent the functioning of the endogenous gene. A glutamine synthase expression system and components thereof are detailed in PCT publications: WO87/04462; WO86/05807; WO89/01036; WO89/10404; and WO91/06657 which are incorporated in their entireties by reference herein. Additionally, glutamine synthase expression vectors that may be used according to the present invention are commercially available from suppliers, including, for example Lonza Biologics, Inc. (Portsmouth, NH). Expression and production of monoclonal antibodies using a GS expression system in murine myeloma cells is described in Bebbington *et al.*, *Bio/technology* 10:169(1992) and in Biblia and Robinson *Biotechnol. Prog.* 11:1 (1995) which are incorporated in their entireties by reference herein.

[0166] The host cell may be co-transfected with two expression vectors of the invention, the first vector encoding a heavy chain derived polypeptide and the second vector encoding a light chain derived polypeptide. The two vectors may contain identical selectable markers which enable equal expression of heavy and light chain polypeptides. Alternatively, a single vector may be used which encodes, and is capable of expressing, both heavy and light chain polypeptides. In such situations, the light chain should be placed before the heavy chain to avoid an excess of toxic free heavy chain (Proudfoot, *Nature* 322:52 (1986); Kohler, *Proc. Natl. Acad. Sci. USA* 77:2197 (1980)). The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA.

[0167] Once an antibody molecule of the invention has been produced by an animal, chemically synthesized, or recombinantly expressed, it may be purified by any method known in the art for purification of an immunoglobulin molecule, for example, by chromatography (e.g., ion exchange, affinity, particularly by affinity for the specific antigen after Protein A, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. In addition, the antibodies of the present invention or fragments thereof can be fused to heterologous

polypeptide sequences described herein or otherwise known in the art, to facilitate purification.

[0168] The present invention encompasses antibodies recombinantly fused or chemically conjugated (including both covalently and non-covalently conjugations) to a polypeptide (or portion thereof, preferably at least 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 amino acids of the polypeptide) of the present invention to generate fusion proteins. The fusion does not necessarily need to be direct, but may occur through linker sequences. The antibodies may be specific for antigens other than polypeptides (or portion thereof, preferably at least 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 amino acids of the polypeptide) of the present invention. For example, antibodies may be used to target the polypeptides of the present invention to particular cell types, either *in vitro* or *in vivo*, by fusing or conjugating the polypeptides of the present invention to antibodies specific for particular cell surface receptors. Antibodies fused or conjugated to the polypeptides of the present invention may also be used in *in vitro* immunoassays and purification methods using methods known in the art. See e.g., Harbor et al., *supra*, and PCT publication WO 93/21232; EP 439,095; Naramura et al., Immunol. Lett. 39:91-99 (1994); U.S. Patent 5,474,981; Gillies et al., PNAS 89:1428-1432 (1992); Fell et al., J. Immunol. 146:2446-2452 (1991), which are incorporated by reference in their entireties.

[0169] The present invention further includes compositions comprising the polypeptides of the present invention fused or conjugated to antibody domains other than the variable regions. For example, the polypeptides of the present invention may be fused or conjugated to an antibody Fc region, or portion thereof. The antibody portion fused to a polypeptide of the present invention may comprise the constant region, hinge region, CH1 domain, CH2 domain, and CH3 domain or any combination of whole domains or portions thereof. The polypeptides may also be fused or conjugated to the above antibody portions to form multimers. For example, Fc portions fused to the polypeptides of the present invention can form dimers through disulfide bonding between the Fc portions. Higher multimeric forms can be made by fusing the polypeptides to portions of IgA and IgM. Methods for fusing or conjugating the polypeptides of the present invention to antibody portions are known in the art. See, e.g., U.S. Patent Nos. 5,336,603; 5,622,929; 5,359,046; 5,349,053; 5,447,851; 5,112,946; EP 307,434; EP 367,166; PCT publications WO 96/04388; WO 91/06570; Ashkenazi et al., Proc. Natl. Acad. Sci. USA 88:10535-10539

(1991); Zheng et al., J. Immunol. 154:5590-5600 (1995); and Vil et al., Proc. Natl. Acad. Sci. USA 89:11337- 11341 (1992) (said references incorporated by reference in their entireties).

[0170] As discussed, *supra*, the polypeptides corresponding to a polypeptide, polypeptide fragment, or a variant of SEQ ID NO:2 may be fused or conjugated to the above antibody portions to increase the *in vivo* half life of the polypeptides or for use in immunoassays using methods known in the art. Further, the polypeptides corresponding to SEQ ID NO:2 may be fused or conjugated to the above antibody portions to facilitate purification. One reported example describes chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins. See EP 394,827; Traunecker et al., Nature 331:84-86 (1988). The polypeptides of the present invention fused or conjugated to an antibody having disulfide- linked dimeric structures (due to the IgG) may also be more efficient in binding and neutralizing other molecules, than the monomeric secreted protein or protein fragment alone. See, for example, Fountoulakis et al., J. Biochem. 270:3958-3964 (1995). In many cases, the Fc part in a fusion protein is beneficial in therapy and diagnosis, and thus can result in, for example, improved pharmacokinetic properties. See, for example, EP A 232,262. Alternatively, deleting the Fc part after the fusion protein has been expressed, detected, and purified, would be desired. For example, the Fc portion may hinder therapy and diagnosis if the fusion protein is used as an antigen for immunizations. In drug discovery, for example, human proteins, such as hIL-5, have been fused with Fc portions for the purpose of high-throughput screening assays to identify antagonists of hIL-5. (See, Bennett et al., J. Molecular Recognition 8:52-58 (1995); Johanson et al., J. Biol. Chem. 270:9459-9471 (1995)).

[0171] Moreover, the antibodies or fragments thereof of the present invention can be fused to marker sequences, such as a peptide to facilitate purification. In preferred embodiments, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311), among others, many of which are commercially available. As described in Gentz et al., Proc. Natl. Acad. Sci. USA 86:821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. Other peptide tags useful for purification

include, but are not limited to, the “HA” tag, which corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson et al., Cell 37:767 (1984)) and the “flag” tag.

[0172] The present invention further encompasses antibodies or fragments thereof conjugated to a diagnostic or therapeutic agent. The antibodies can be used diagnostically to, for example, monitor the development or progression of a tumor as part of a clinical testing procedure to, e.g., determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, radioactive materials, positron emitting metals using various positron emission tomographies, and nonradioactive paramagnetic metal ions. The detectable substance may be coupled or conjugated either directly to the antibody (or fragment thereof) or indirectly, through an intermediate (such as, for example, a linker known in the art) using techniques known in the art. See, for example, U.S. Patent No. 4,741,900 for metal ions which can be conjugated to antibodies for use as diagnostics according to the present invention.

[0173] Further, an antibody or fragment thereof may be conjugated to a therapeutic moiety such as a cytotoxin, e.g., a cytostatic or cytotoxic agent, a therapeutic agent or a radioactive metal ion, e.g., alpha-emitters such as, for example, ^{213}Bi . A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include paclitaxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, teniposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis- dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin,

mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine and vinblastine).

[0174] The conjugates of the invention can be used for modifying a given biological response, the therapeutic agent or drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor, α -interferon, β -interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator, an apoptotic agent, e.g., TNF-alpha, TNF-beta, AIM I (See, International Publication No. WO 97/33899), AIM II (See, International Publication No. WO 97/34911), Fas Ligand (Takahashi *et al.*, *Int. Immunol.*, 6:1567-1574 (1994)), VEGI (See, International Publication No. WO 99/23105), a thrombotic agent or an anti-angiogenic agent, e.g., angiostatin or endostatin; or, biological response modifiers such as, for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-CSF"), granulocyte colony stimulating factor ("G-CSF"), or other growth factors.

[0175] Antibodies may also be attached to solid supports, which are particularly useful for immunoassays or purification of the target antigen. Such solid supports include, but are not limited to, glass, cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene.

[0176] Techniques for conjugating such therapeutic moiety to antibodies are well known. See, for example., Arnon et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in *Monoclonal Antibodies And Cancer Therapy*, Reisfeld et al. (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom et al., "Antibodies For Drug Delivery", in *Controlled Drug Delivery* (2nd Ed.), Robinson et al. (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in *Monoclonal Antibodies '84: Biological And Clinical Applications*, Pinchera et al. (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in *Monoclonal Antibodies For Cancer Detection And Therapy*, Baldwin et al. (eds.), pp. 303-16 (Academic Press 1985), and Thorpe et al., "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", *Immunol. Rev.* 62:119-58 (1982).

[0177] Alternatively, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate as described by Segal in U.S. Patent No. 4,676,980, which is incorporated herein by reference in its entirety.

[0178] An antibody, with or without a therapeutic moiety conjugated to it, administered alone or in combination with cytotoxic factor(s) and/or cytokine(s) can be used as a therapeutic.

Immunophenotyping

[0179] The antibodies of the invention may be utilized for immunophenotyping of cell lines and biological samples. Translation products of the genes of the present invention may be useful as cell specific markers, or more specifically as cellular markers that are differentially expressed at various stages of differentiation and/or maturation of particular cell types. Monoclonal antibodies directed against a specific epitope, or combination of epitopes, will allow for the screening of cellular populations expressing the marker. Various techniques can be utilized using monoclonal antibodies to screen for cellular populations expressing the marker(s), and include magnetic separation using antibody-coated magnetic beads, “panning” with antibody attached to a solid matrix (i.e., plate), and flow cytometry (See, e.g., U.S. Patent 5,985,660; and Morrison *et al.*, *Cell*, 96:737-49 (1999)).

[0180] These techniques allow for the screening of particular populations of cells, such as might be found with hematological malignancies (i.e. minimal residual disease (MRD) in acute leukemic patients) and “non-self” cells in transplantations to prevent Graft-versus-Host Disease (GVHD). Alternatively, these techniques allow for the screening of hematopoietic stem and progenitor cells capable of undergoing proliferation and/or differentiation, as might be found in human umbilical cord blood.

Assays For Antibody Binding

[0181] The antibodies of the invention may be assayed for immunospecific binding by any method known in the art. The immunoassays which can be used include but are not limited to competitive and non-competitive assay systems using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), “sandwich” immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion

precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, and protein A immunoassays, to name but a few. Such assays are routine and well known in the art (see, e.g., Ausubel et al, eds, 1994, *Current Protocols in Molecular Biology*, Vol. 1, John Wiley & Sons, Inc., New York, which is incorporated by reference herein in its entirety). Exemplary immunoassays are described briefly below (but are not intended by way of limitation).

[0182] Immunoprecipitation protocols generally comprise lysing a population of cells in a lysis buffer such as RIPA buffer (1% NP-40 or Triton X- 100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate at pH 7.2, 1% Trasylol) supplemented with protein phosphatase and/or protease inhibitors (e.g., EDTA, PMSF, aprotinin, sodium vanadate), adding the antibody of interest to the cell lysate, incubating for a period of time (e.g., 1-4 hours) at 4° C, adding protein A and/or protein G sepharose beads to the cell lysate, incubating for about an hour or more at 4° C, washing the beads in lysis buffer and resuspending the beads in SDS/sample buffer. The ability of the antibody of interest to immunoprecipitate a particular antigen can be assessed by, e.g., western blot analysis. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the binding of the antibody to an antigen and decrease the background (e.g., pre-clearing the cell lysate with sepharose beads). For further discussion regarding immunoprecipitation protocols see, e.g., Ausubel et al., eds., (1994), *Current Protocols in Molecular Biology*, Vol. 1, John Wiley & Sons, Inc., New York, section 10.16.1.

[0183] Western blot analysis generally comprises preparing protein samples, electrophoresis of the protein samples in a polyacrylamide gel (e.g., 8%- 20% SDS-PAGE depending on the molecular weight of the antigen), transferring the protein sample from the polyacrylamide gel to a membrane such as nitrocellulose, PVDF or nylon, blocking the membrane in blocking solution (e.g., PBS with 3% BSA or non-fat milk), washing the membrane in washing buffer (e.g., PBS-Tween 20), blocking the membrane with primary antibody (the antibody of interest) diluted in blocking buffer, washing the membrane in washing buffer, blocking the membrane with a secondary antibody (which recognizes the primary antibody, e.g., an anti-human antibody) conjugated to an enzymatic substrate (e.g., horseradish peroxidase or alkaline phosphatase) or radioactive molecule (e.g., ³²P or ¹²⁵I) diluted in blocking buffer, washing the membrane in wash buffer, and detecting the

presence of the antigen. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected and to reduce the background noise. For further discussion regarding western blot protocols see, e.g., Ausubel et al., eds., (1994), *Current Protocols in Molecular Biology*, Vol. 1, John Wiley & Sons, Inc., New York, section 10.8.1.

[0184] ELISAs comprise preparing antigen, coating the well of a 96 well microtiter plate with the antigen, adding the antibody of interest conjugated to a detectable compound such as an enzymatic substrate (e.g., horseradish peroxidase or alkaline phosphatase) to the well and incubating for a period of time, and detecting the presence of the antigen. In ELISAs the antibody of interest does not have to be conjugated to a detectable compound; instead, a second antibody (which recognizes the antibody of interest) conjugated to a detectable compound may be added to the well. Further, instead of coating the well with the antigen, the antibody may be coated to the well. In this case, a second antibody conjugated to a detectable compound may be added following the addition of the antigen of interest to the coated well. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected as well as other variations of ELISAs known in the art. For further discussion regarding ELISAs see, e.g., Ausubel et al., eds., (1994), *Current Protocols in Molecular Biology*, Vol. 1, John Wiley & Sons, Inc., New York, section 11.2.1.

[0185] The binding affinity of an antibody to an antigen and the off-rate of an antibody-antigen interaction can be determined by competitive binding assays. One example of a competitive binding assay is a radioimmunoassay comprising the incubation of labeled antigen (e.g., ³H or ¹²⁵I) with the antibody of interest in the presence of increasing amounts of unlabeled antigen, and the detection of the antibody bound to the labeled antigen. The affinity of the antibody of interest for a particular antigen and the binding off-rates can be determined from the data by scatchard plot analysis. Competition with a second antibody can also be determined using radioimmunoassays. In this case, the antigen is incubated with antibody of interest conjugated to a labeled compound (e.g., ³H or ¹²⁵I) in the presence of increasing amounts of an unlabeled second antibody.

Therapeutic Uses

[0186] The present invention is further directed to antibody-based therapies which involve administering antibodies of the invention to an animal, preferably a mammal, and most preferably a human, patient for treating one or more of the disclosed diseases, disorders, or conditions. Therapeutic compounds of the invention include, but are not limited to, antibodies of the invention (including fragments, analogs and derivatives thereof as described herein) and nucleic acids encoding antibodies of the invention (including fragments, analogs and derivatives thereof and anti-idiotypic antibodies as described herein). The antibodies of the invention can be used to treat, inhibit or prevent diseases, disorders or conditions associated with aberrant expression and/or activity of a polypeptide of the invention, including, but not limited to, any one or more of the diseases, disorders, or conditions described herein. The treatment and/or prevention of diseases, disorders, or conditions associated with aberrant expression and/or activity of a polypeptide of the invention includes, but is not limited to, alleviating symptoms associated with those diseases, disorders or conditions. Antibodies of the invention may be provided in pharmaceutically acceptable compositions as known in the art or as described herein.

[0187] A summary of the ways in which the antibodies of the present invention may be used therapeutically includes binding polynucleotides or polypeptides of the present invention locally or systemically in the body or by direct cytotoxicity of the antibody, e.g. as mediated by complement (CDC) or by effector cells (ADCC). Some of these approaches are described in more detail below. Armed with the teachings provided herein, one of ordinary skill in the art will know how to use the antibodies of the present invention for diagnostic, monitoring or therapeutic purposes without undue experimentation.

[0188] The antibodies of this invention may be advantageously utilized in combination with other monoclonal or chimeric antibodies, or with lymphokines or hematopoietic growth factors (such as, e.g., IL-2, IL-3 and IL-7), for example, which serve to increase the number or activity of effector cells which interact with the antibodies.

[0189] The antibodies of the invention may be administered alone or in combination with other types of treatments (e.g., radiation therapy, chemotherapy, hormonal therapy, immunotherapy and anti-tumor agents). In preferred embodiments, the antibodies of the invention are administered in combination with therapy directed toward treating or preventing cell injury (e.g., neural injury) associated with stroke and/or hypoxia.

Generally, administration of products of a species origin or species reactivity (in the case of antibodies) that is the same species as that of the patient is preferred. Thus, in a preferred embodiment, human antibodies, fragments derivatives, analogs, or nucleic acids, are administered to a human patient for therapy or prophylaxis.

[0190] It is preferred to use high affinity and/or potent *in vivo* inhibiting and/or neutralizing antibodies against polypeptides or polynucleotides of the present invention, fragments or regions thereof, for both immunoassays directed to and therapy of disorders related to polynucleotides or polypeptides, including fragments thereof, of the present invention. Such antibodies, fragments, or regions, will preferably have an affinity for polynucleotides or polypeptides of the invention, including fragments thereof. Preferred binding affinities include those with a dissociation constant or K_d less than 5×10^{-2} M, 10^{-2} M, 5×10^{-3} M, 10^{-3} M, 5×10^{-4} M, 10^{-4} M, 5×10^{-5} M, 10^{-5} M, 5×10^{-6} M, 10^{-6} M, 5×10^{-7} M, 10^{-7} M, 5×10^{-8} M, 10^{-8} M, 5×10^{-9} M, 10^{-9} M, 5×10^{-10} M, 10^{-10} M, 5×10^{-11} M, 10^{-11} M, 5×10^{-12} M, 10^{-12} M, 5×10^{-13} M, 10^{-13} M, 5×10^{-14} M, 10^{-14} M, 5×10^{-15} M, and 10^{-15} M.

Gene Therapy

[0191] In a specific embodiment, nucleic acids comprising sequences encoding antibodies or functional derivatives thereof, are administered to treat, inhibit or prevent a disease or disorder associated with aberrant expression and/or activity of a polypeptide of the invention, by way of gene therapy. Gene therapy refers to therapy performed by the administration to a subject of an expressed or expressible nucleic acid. In this embodiment of the invention, the nucleic acids produce their encoded protein that mediates a therapeutic effect.

[0192] Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

[0193] For general reviews of the methods of gene therapy, see Goldspiel et al., *Clinical Pharmacy* 12:488-505 (1993); Wu and Wu, *Biotherapy* 3:87-95 (1991); Tolstoshev, *Ann. Rev. Pharmacol. Toxicol.* 32:573-596 (1993); Mulligan, *Science* 260:926-932 (1993); and Morgan and Anderson, *Ann. Rev. Biochem.* 62:191-217 (1993); May, *TIBTECH* 11(5):155-215 (1993). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), *Current Protocols in*

Molecular Biology, John Wiley & Sons, NY (1993); and Kriegler, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY (1990).

[0194] In a preferred embodiment, the compound comprises nucleic acid sequences encoding an antibody, said nucleic acid sequences being part of expression vectors that express the antibody or fragments or chimeric proteins or heavy or light chains thereof in a suitable host. In particular, such nucleic acid sequences have promoters operably linked to the antibody coding region, said promoter being inducible or constitutive, and, optionally, tissue-specific. In another particular embodiment, nucleic acid molecules are used in which the antibody coding sequences and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the antibody encoding nucleic acids (Koller and Smithies, Proc. Natl. Acad. Sci. USA 86:8932-8935 (1989); Zijlstra et al., Nature 342:435-438 (1989). In specific embodiments, the expressed antibody molecule is a single chain antibody; alternatively, the nucleic acid sequences include sequences encoding both the heavy and light chains, or fragments thereof, of the antibody.

[0195] Delivery of the nucleic acids into a patient may be either direct, in which case the patient is directly exposed to the nucleic acid or nucleic acid- carrying vectors, or indirect, in which case, cells are first transformed with the nucleic acids *in vitro*, then transplanted into the patient. These two approaches are known, respectively, as *in vivo* or *ex vivo* gene therapy.

[0196] In a specific embodiment, the nucleic acid sequences are directly administered *in vivo*, where it is expressed to produce the encoded product. This can be accomplished by any of numerous methods known in the art, e.g., by constructing them as part of an appropriate nucleic acid expression vector and administering it so that they become intracellular, e.g., by infection using defective or attenuated retrovirals or other viral vectors (see U.S. Patent No. 4,980,286), or by direct injection of naked DNA, or by use of microparticle bombardment (e.g., a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, encapsulation in liposomes, microparticles, or microcapsules, or by administering them in linkage to a peptide which is known to enter the nucleus, by administering it in linkage to a ligand subject to receptor-mediated endocytosis (see, e.g., Wu and Wu, J. Biol. Chem. 262:4429-4432 (1987)) (which can be used to target cell types specifically expressing the receptors), etc. In another

embodiment, nucleic acid-ligand complexes can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (see, e.g., PCT Publications WO 92/06180; WO 92/22635; WO92/20316; WO93/14188, WO 93/20221). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination (Koller and Smithies, Proc. Natl. Acad. Sci. USA 86:8932-8935 (1989); Zijlstra et al., Nature 342:435-438 (1989)).

[0197] In a specific embodiment, viral vectors that contains nucleic acid sequences encoding an antibody of the invention are used. For example, a retroviral vector can be used (see Miller et al., Meth. Enzymol. 217:581-599 (1993)). These retroviral vectors contain the components necessary for the correct packaging of the viral genome and integration into the host cell DNA. The nucleic acid sequences encoding the antibody to be used in gene therapy are cloned into one or more vectors, which facilitates delivery of the gene into a patient. More detail about retroviral vectors can be found in Boesen et al., Biotherapy 6:291-302 (1994), which describes the use of a retroviral vector to deliver the *mdr1* gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are: Clowes et al., J. Clin. Invest. 93:644-651 (1994); Kiem et al., Blood 83:1467-1473 (1994); Salmons and Gunzberg, Human Gene Therapy 4:129-141 (1993); and Grossman and Wilson, Curr. Opin. in Genetics and Devel. 3:110-114 (1993).

[0198] Adenoviruses are other viral vectors that can be used in gene therapy. Adenoviruses are especially attractive vehicles for delivering genes to respiratory epithelia. Adenoviruses naturally infect respiratory epithelia where they cause a mild disease. Other targets for adenovirus-based delivery systems are liver, the central nervous system, endothelial cells, and muscle. Adenoviruses have the advantage of being capable of infecting non-dividing cells. Kozarsky and Wilson, Current Opinion in Genetics and Development 3:499-503 (1993) present a review of adenovirus-based gene therapy. Bout et al., Human Gene Therapy 5:3-10 (1994) demonstrated the use of adenovirus vectors to transfer genes to the respiratory epithelia of rhesus monkeys. Other instances of the use of adenoviruses in gene therapy can be found in Rosenfeld et al., Science 252:431-434 (1991); Rosenfeld et al., Cell 68:143- 155 (1992); Mastrangeli et al., J. Clin. Invest.

91:225-234 (1993); PCT Publication WO94/12649; and Wang, et al., Gene Therapy 2:775-783 (1995). In a preferred embodiment, adenovirus vectors are used.

[0199] Adeno-associated virus (AAV) has also been proposed for use in gene therapy (Walsh et al., Proc. Soc. Exp. Biol. Med. 204:289-300 (1993); U.S. Patent No. 5,436,146).

[0200] Another approach to gene therapy involves transferring a gene to cells in tissue culture by such methods as electroporation, lipofection, calcium phosphate mediated transfection, or viral infection. Usually, the method of transfer includes the transfer of a selectable marker to the cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a patient.

[0201] In this embodiment, the nucleic acid is introduced into a cell prior to administration *in vivo* of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including but not limited to transfection, electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequences, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene transfer, spheroplast fusion, etc. Numerous techniques are known in the art for the introduction of foreign genes into cells (see, e.g., Loeffler and Behr, Meth. Enzymol. 217:599-618 (1993); Cohen et al., Meth. Enzymol. 217:618-644 (1993); Cline, Pharmac. Ther. 29:69-92m (1985) and may be used in accordance with the present invention, provided that the necessary developmental and physiological functions of the recipient cells are not disrupted. The technique should provide for the stable transfer of the nucleic acid to the cell, so that the nucleic acid is expressible by the cell and preferably heritable and expressible by its cell progeny.

[0202] The resulting recombinant cells can be delivered to a patient by various methods known in the art. Recombinant blood cells (e.g., hematopoietic stem or progenitor cells) are preferably administered intravenously. The amount of cells envisioned for use depends on the desired effect, patient state, etc., and can be determined by one skilled in the art.

[0203] Cells into which a nucleic acid can be introduced for purposes of gene therapy encompass any desired, available cell type, and include but are not limited to epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes; blood cells such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils,

eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells, e.g., as obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, etc.

[0204] In a preferred embodiment, the cell used for gene therapy is autologous to the patient.

[0205] In an embodiment in which recombinant cells are used in gene therapy, nucleic acid sequences encoding an antibody are introduced into the cells such that they are expressible by the cells or their progeny, and the recombinant cells are then administered *in vivo* for therapeutic effect. In a specific embodiment, stem or progenitor cells are used. Any stem and/or progenitor cells which can be isolated and maintained *in vitro* can potentially be used in accordance with this embodiment of the present invention (see e.g. PCT Publication WO 94/08598; Stemple and Anderson, Cell 71:973-985 (1992); Rheinwald, Meth. Cell Bio. 21A:229 (1980); and Pittelkow and Scott, Mayo Clinic Proc. 61:771 (1986)).

[0206] In a specific embodiment, the nucleic acid to be introduced for purposes of gene therapy comprises an inducible promoter operably linked to the coding region, such that expression of the nucleic acid is controllable by the presence or absence of an appropriate inducer of transcription.

Demonstration of Therapeutic or Prophylactic Activity

[0207] The compounds or pharmaceutical compositions of the invention are preferably tested *in vitro*, and then *in vivo* for the desired therapeutic or prophylactic activity, prior to use in humans. For example, *in vitro* assays to demonstrate the therapeutic or prophylactic utility of a compound or pharmaceutical composition include, the effect of a compound on a cell line or a patient tissue sample. The effect of the compound or composition on the cell line and/or tissue sample can be determined utilizing techniques known to those of skill in the art including, but not limited to, rosette formation assays and cell lysis assays. In accordance with the invention, *in vitro* assays which can be used to determine whether administration of a specific compound is indicated, include *in vitro* cell culture assays in which a patient tissue sample is grown in culture, and exposed to or otherwise administered a compound, and the effect of such compound upon the tissue sample is observed.

Therapeutic/Prophylactic Administration and Composition

[0208] The invention provides methods of treatment, inhibition and prophylaxis by administration to a subject of an effective amount of a compound or pharmaceutical composition of the invention, preferably a polypeptide or antibody of the invention. In a preferred embodiment, the compound is substantially purified (e.g., substantially free from substances that limit its effect or produce undesired side-effects). The subject is preferably an animal, including but not limited to animals such as cows, pigs, horses, chickens, cats, dogs, etc., and is preferably a mammal, and most preferably human.

[0209] Formulations and methods of administration that can be employed when the compound comprises a nucleic acid or an immunoglobulin are described above; additional appropriate formulations and routes of administration can be selected from among those described herein below.

[0210] Various delivery systems are known and can be used to administer a compound of the invention, e.g., encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the compound, receptor-mediated endocytosis (see, e.g., Wu and Wu, J. Biol. Chem. 262:4429-4432 (1987)), construction of a nucleic acid as part of a retroviral or other vector, etc. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds or compositions may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compounds or compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, e.g., by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

[0211] In a specific embodiment, it may be desirable to administer the pharmaceutical compounds or compositions of the invention locally to the area in need of treatment; this

may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, e.g., in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. Preferably, when administering a protein, including an antibody, of the invention, care must be taken to use materials to which the protein does not absorb.

[0212] In another embodiment, the compound or composition can be delivered in a vesicle, in particular a liposome (see Langer, *Science* 249:1527-1533 (1990); Treat et al., in *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353- 365 (1989); Lopez-Berestein, *ibid.*, pp. 317-327; see generally *ibid.*)

[0213] In yet another embodiment, the compound or composition can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, *supra*; Sefton, *CRC Crit. Ref. Biomed. Eng.* 14:201 (1987); Buchwald et al., *Surgery* 88:507 (1980); Saudek et al., *N. Engl. J. Med.* 321:574 (1989)). In another embodiment, polymeric materials can be used (see *Medical Applications of Controlled Release*, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); *Controlled Drug Bioavailability, Drug Product Design and Performance*, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, J., *Macromol. Sci. Rev. Macromol. Chem.* 23:61 (1983); see also Levy et al., *Science* 228:190 (1985); During et al., *Ann. Neurol.* 25:351 (1989); Howard et al., *J.Neurosurg.* 71:105 (1989)). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, e.g., the brain, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in *Medical Applications of Controlled Release*, *supra*, vol. 2, pp. 115-138 (1984)). Other controlled release systems are discussed in the review by Langer (*Science* 249:1527-1533 (1990)).

[0214] In a specific embodiment where the compound of the invention is a nucleic acid encoding a protein, the nucleic acid can be administered *in vivo* to promote expression of its encoded protein, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, e.g., by use of a retroviral vector (see U.S. Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (e.g., a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface

receptors or transfecting agents, or by administering it in linkage to a homeobox- like peptide which is known to enter the nucleus (see e.g., Joliot et al., Proc. Natl. Acad. Sci. USA 88:1864-1868 (1991)), etc. Alternatively, a nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

[0215] The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a compound, and a pharmaceutically acceptable carrier. In a specific embodiment, the term “pharmaceutically acceptable” means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term “carrier” refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in “Remington's Pharmaceutical Sciences” by E.W. Martin. Such compositions will contain a therapeutically effective amount of the compound, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

[0216] In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

[0217] The compounds of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with anions such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with cations such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

[0218] The amount of the compound of the invention which will be effective in the treatment, inhibition and prevention of a disease or disorder associated with aberrant expression and/or activity of a polypeptide of the invention can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

[0219] For antibodies, the dosage administered to a patient is typically 0.1 mg/kg to 100 mg/kg of the patient's body weight. Preferably, the dosage administered to a patient is between 0.1 mg/kg and 20 mg/kg of the patient's body weight, more preferably 1 mg/kg to 10 mg/kg of the patient's body weight. Generally, human antibodies have a longer half-life within the human body than antibodies from other species due to the immune response

to the foreign polypeptides. Thus, lower dosages of human antibodies and less frequent administration is often possible. Further, the dosage and frequency of administration of antibodies of the invention may be reduced by enhancing uptake and tissue penetration (e.g., into the brain) of the antibodies by modifications such as, for example, lipidation.

[0220] The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

Diagnosis and Imaging

[0221] Labeled antibodies, and derivatives and analogs thereof, which specifically bind to a polypeptide of interest can be used for diagnostic purposes to detect, diagnose, or monitor diseases, disorders, and/or conditions associated with the aberrant expression and/or activity of a polypeptide of the invention. The invention provides for the detection of aberrant expression of a polypeptide of interest, comprising (a) assaying the expression of the polypeptide of interest in cells or body fluid of an individual using one or more antibodies specific to the polypeptide interest and (b) comparing the level of gene expression with a standard gene expression level, whereby an increase or decrease in the assayed polypeptide gene expression level compared to the standard expression level is indicative of aberrant expression.

[0222] The invention provides a diagnostic assay for diagnosing a disorder, comprising (a) assaying the expression of the polypeptide of interest in cells or body fluid of an individual using one or more antibodies specific to the polypeptide interest and (b) comparing the level of gene expression with a standard gene expression level, whereby an increase or decrease in the assayed polypeptide gene expression level compared to the standard expression level is indicative of a particular disorder. With respect to cancer, the presence of a relatively high amount of transcript in biopsied tissue from an individual may indicate a predisposition for the development of the disease, or may provide a means for detecting the disease prior to the appearance of actual clinical symptoms. A more definitive diagnosis of this type may allow health professionals to employ preventative

measures or aggressive treatment earlier thereby preventing the development or further progression of the cancer.

[0223] Antibodies of the invention can be used to assay protein levels in a biological sample using classical immunohistological methods known to those of skill in the art (e.g., see Jalkanen et al., J. Cell. Biol. 101:976-985 (1985); Jalkanen et al., J. Cell . Biol. 105:3087-3096 (1987)). Other antibody-based methods useful for detecting protein gene expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA). Suitable antibody assay labels are known in the art and include enzyme labels, such as, glucose oxidase; radioisotopes, such as iodine (¹²⁵I, ¹²¹I), carbon (¹⁴C), sulfur (³⁵S), tritium (³H), indium (¹¹²In), and technetium (⁹⁹Tc); luminescent labels, such as luminol; and fluorescent labels, such as fluorescein and rhodamine, and biotin.

[0224] One facet of the invention is the detection and diagnosis of a disease or disorder associated with aberrant expression of a polypeptide of interest in an animal, preferably a mammal and most preferably a human. In one embodiment, diagnosis comprises: a) administering (for example, parenterally, subcutaneously, or intraperitoneally) to a subject an effective amount of a labeled molecule which specifically binds to the polypeptide of interest; b) waiting for a time interval following the administering for permitting the labeled molecule to preferentially concentrate at sites in the subject where the polypeptide is expressed (and for unbound labeled molecule to be cleared to background level); c) determining background level; and d) detecting the labeled molecule in the subject, such that detection of labeled molecule above the background level indicates that the subject has a particular disease or disorder associated with aberrant expression of the polypeptide of interest. Background level can be determined by various methods including, comparing the amount of labeled molecule detected to a standard value previously determined for a particular system.

[0225] It will be understood in the art that the size of the subject and the imaging system used will determine the quantity of imaging moiety needed to produce diagnostic images. In the case of a radioisotope moiety, for a human subject, the quantity of radioactivity injected will normally range from about 5 to 20 millicuries of ^{99m}Tc. The labeled antibody or antibody fragment will then preferentially accumulate at the location of cells which contain the specific protein. *In vivo* tumor imaging is described in S.W. Burchiel et

al., "Immunopharmacokinetics of Radiolabeled Antibodies and Their Fragments." (Chapter 13 in Tumor Imaging: The Radiochemical Detection of Cancer, S.W. Burchiel and B. A. Rhodes, eds., Masson Publishing Inc. (1982)).

[0226] Depending on several variables, including the type of label used and the mode of administration, the time interval following the administration for permitting the labeled molecule to preferentially concentrate at sites in the subject and for unbound labeled molecule to be cleared to background level is 6 to 48 hours or 6 to 24 hours or 6 to 12 hours. In another embodiment the time interval following administration is 5 to 20 days or 5 to 10 days.

[0227] In an embodiment, monitoring of the disease or disorder is carried out by repeating the method for diagnosing the disease or disease, for example, one month after initial diagnosis, six months after initial diagnosis, one year after initial diagnosis, etc.

[0228] Presence of the labeled molecule can be detected in the patient using methods known in the art for *in vivo* scanning. These methods depend upon the type of label used. Skilled artisans will be able to determine the appropriate method for detecting a particular label. Methods and devices that may be used in the diagnostic methods of the invention include, but are not limited to, computed tomography (CT), whole body scan such as position emission tomography (PET), magnetic resonance imaging (MRI), and sonography.

[0229] In a specific embodiment, the molecule is labeled with a radioisotope and is detected in the patient using a radiation responsive surgical instrument (Thurston et al., U.S. Patent No. 5,441,050). In another embodiment, the molecule is labeled with a fluorescent compound and is detected in the patient using a fluorescence responsive scanning instrument. In another embodiment, the molecule is labeled with a positron emitting metal and is detected in the patient using positron emission-tomography. In yet another embodiment, the molecule is labeled with a paramagnetic label and is detected in a patient using magnetic resonance imaging (MRI).

Kits

[0230] The present invention provides kits that can be used in the above methods. In one embodiment, a kit comprises an antibody of the invention, preferably a purified antibody, in one or more containers. In a specific embodiment, the kits of the present

invention contain a substantially isolated polypeptide comprising an epitope which is specifically immunoreactive with an antibody included in the kit. Preferably, the kits of the present invention further comprise a control antibody which does not react with the polypeptide of interest. In another specific embodiment, the kits of the present invention contain a means for detecting the binding of an antibody to a polypeptide of interest (e.g., the antibody may be conjugated to a detectable substrate such as a fluorescent compound, an enzymatic substrate, a radioactive compound or a luminescent compound, or a second antibody which recognizes the first antibody may be conjugated to a detectable substrate).

[0231] In another specific embodiment of the present invention, the kit is a diagnostic kit for use in screening serum containing antibodies specific against proliferative and/or cancerous polynucleotides and polypeptides. Such a kit may include a control antibody that does not react with the polypeptide of interest. Such a kit may include a substantially isolated polypeptide comprising an epitope which is specifically immunoreactive with at least one anti-polypeptide antibody. Further, such a kit includes means for detecting the binding of said antibody to the polypeptide (e.g., the antibody may be conjugated to a fluorescent compound such as fluorescein or rhodamine which can be detected by flow cytometry). In specific embodiments, the kit may include a recombinantly produced or chemically synthesized polypeptide. The polypeptide of the kit may also be attached to a solid support.

[0232] In a more specific embodiment the detecting means of the above-described kit includes a solid support to which said polypeptide is attached. Such a kit may also include a non-attached reporter-labeled anti-human antibody. In this embodiment, binding of the antibody to the polypeptide can be detected by binding of the said reporter-labeled antibody.

[0233] In an additional embodiment, the invention includes a diagnostic kit for use in screening serum containing antigens of the polypeptide of the invention. The diagnostic kit includes a substantially isolated antibody specifically immunoreactive with a polypeptide or polynucleotide of the invention, and means for detecting the binding of the polynucleotide or polypeptide to the antibody. In one embodiment, the antibody is attached to a solid support. In a specific embodiment, the antibody may be a monoclonal antibody. The detecting means of the kit may include a second, labeled monoclonal antibody.

Alternatively, or in addition, the detecting means may include a labeled, competing polypeptide.

[0234] In one diagnostic configuration, test serum is reacted with a solid phase reagent having a surface-bound polypeptide obtained by the methods of the present invention. After binding the polypeptide-specific antibody to the reagent and removing unbound serum components by washing, the reagent is reacted with reporter-labeled anti-human antibody to bind reporter to the reagent in proportion to the amount of bound anti-polypeptide antibody on the solid support. The reagent is again washed to remove unbound labeled antibody, and the amount of reporter associated with the reagent is determined. Typically, the reporter is an enzyme which is detected by incubating the solid phase in the presence of a suitable fluorometric, luminescent or colorimetric substrate (Sigma, St. Louis, MO).

[0235] The solid surface reagent in the above assay is prepared by known techniques for attaching protein material to solid support material, such as polymeric beads, dip sticks, 96-well plate or filter material. These attachment methods generally include non-specific adsorption of the protein to the support or covalent attachment of the protein, typically through a free amine group, to a chemically reactive group on the solid support, such as an activated carboxyl, hydroxyl, or aldehyde group. Alternatively, streptavidin coated plates can be used in conjunction with biotinylated antigen(s).

[0236] Thus, the invention provides an assay system or kit for carrying out this diagnostic method. The kit generally includes a support with surface-bound recombinant antigens, and a reporter-labeled anti-human antibody for detecting surface-bound anti-antigen antibody.

Fusion Proteins

[0237] Any stanniocalcin polypeptide can be used to generate fusion proteins. For example, the stanniocalcin polypeptide, when fused to a second protein, can be used as an antigenic tag. Antibodies raised against the stanniocalcin polypeptide can be used to indirectly detect the second protein by binding to the stanniocalcin. Moreover, because secreted proteins target cellular locations based on trafficking signals, the stanniocalcin polypeptides can be used as a targeting molecule once fused to other proteins.

[0238] Examples of domains that can be fused to stanniocalcin polypeptides include not only heterologous signal sequences, but also other heterologous functional regions. The fusion does not necessarily need to be direct, but may occur through linker sequences.

[0239] In certain preferred embodiments, stanniocalcin proteins of the invention comprise fusion proteins wherein the stanniocalcin polypeptides are those described above as m-n. Polynucleotides encoding these fusion proteins are also encompassed by the invention, as are antibodies that bind one or more of these fusion proteins. Moreover, variants of these fusion proteins (e.g., polypeptides at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to these fusion proteins and polypeptides encoded by the polynucleotide which hybridizes, under stringent conditions, to the polynucleotide encoding these fusion proteins, or the complement thereof) are encompassed by the invention. Antibodies that bind these variants of the invention are also encompassed by the invention. Polynucleotides encoding these variants are also encompassed by the invention.

[0240] Moreover, fusion proteins may also be engineered to improve characteristics of the stanniocalcin polypeptide. For instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the stanniocalcin polypeptide to improve stability and persistence during purification from the host cell or subsequent handling and storage. Also, peptide moieties may be added to the stanniocalcin polypeptide to facilitate purification. Such regions may be removed prior to final preparation of the stanniocalcin polypeptide. The addition of peptide moieties to facilitate handling of polypeptides are familiar and routine techniques in the art.

[0241] Moreover, stanniocalcin polypeptides, including fragments, and specifically epitopes, can be combined with parts of the constant domain of immunoglobulins (IgG), resulting in chimeric polypeptides. These fusion proteins facilitate purification and show an increased half-life in vivo. One reported example describes chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins. (EP A 394,827; Traunecker *et al.*, *Nature*, 331:84-86 (1988).) Fusion proteins having disulfide-linked dimeric structures (due to the IgG) can also be more efficient in binding and neutralizing other molecules, than the monomeric secreted protein or protein fragment alone. (Fountoulakis *et al.*, *J. Biochem.*, 270:3958-64 (1995).)

[0242] Similarly, EP-A-O 464 533 (Canadian counterpart 2045869) discloses fusion proteins comprising various portions of constant region of immunoglobulin molecules together with another human protein or part thereof. In many cases, the Fc part in a fusion protein is beneficial in therapy and diagnosis, and thus can result in, for example, improved pharmacokinetic properties. (EP-A 0232 262.) Alternatively, deleting the Fc part after the fusion protein has been expressed, detected, and purified, would be desired. For example, the Fc portion may hinder therapy and diagnosis if the fusion protein is used as an antigen for immunizations. In drug discovery, for example, human proteins, such as hIL-5, have been fused with Fc portions for the purpose of high-throughput screening assays to identify antagonists of hIL-5. (See, Bennett *et al.*, *J. Molecular Recognition*, 8:52-58 (1995); Johanson *et al.*, *J. Biol. Chem.*, 270:9459-71 (1995).)

[0243] Additionally, as discussed herein, polypeptides and/or antibodies of the present invention (including fragments or variants thereof) may be fused with albumin (including but not limited to, recombinant human serum albumin or fragments or variants thereof (see, e.g., U.S. Patent No. 5,876,969, issued March 2, 1999, EP Patent 0 413 622, and U.S. Patent No. 5,766,883, issued June 16, 1998, herein incorporated by reference in their entirety)). In a preferred embodiment, polypeptides and/or antibodies of the present invention (including fragments or variants thereof) are fused with the mature form of human serum albumin (i.e., amino acids 1 – 585 of human serum albumin as shown in Figures 1 and 2 of EP Patent 0 322 094) which is herein incorporated by reference in its entirety. In another preferred embodiment, polypeptides and/or antibodies of the present invention (including fragments or variants thereof) are fused with polypeptide fragments comprising, or alternatively consisting of, amino acid residues 1-z of human serum albumin, where z is an integer from 369 to 419, as described in U.S. Patent 5,766,883 herein incorporated by reference in its entirety. Polypeptides and/or antibodies of the present invention (including fragments or variants thereof) may be fused to either the N- or C-terminal end of the heterologous protein (e.g., immunoglobulin Fc polypeptide or human serum albumin polypeptide). Polynucleotides encoding fusion proteins of the invention are also encompassed by the invention.

[0244] Moreover, the stanniocalcin polypeptides can be fused to marker sequences, such as a peptide which facilitates purification of stanniocalcin. In preferred embodiments, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE

vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311), among others, many of which are commercially available. As described in Gentz et al., Proc. Natl. Acad. Sci. USA 86:821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. Another peptide tag useful for purification, the "HA" tag, corresponds to an epitope derived from the influenza hemagglutinin protein. (Wilson et al., Cell 37:767 (1984).)

[0245] Thus, any of these above fusions can be engineered using the stanniocalcin polynucleotides or polypeptides.

Vectors, Host Cells, and Protein Production

[0246] The present invention also relates to vectors containing the stanniocalcin polynucleotides, host cells, and the production of polypeptides by recombinant techniques. The vector may be, for example, a phage, plasmid, viral, or retroviral vector. Retroviral vectors may be replication competent or replication defective. In the latter case, viral propagation generally will occur only in complementing host cells.

[0247] Stanniocalcin polynucleotides may be joined to a vector containing a selectable marker for propagation in a host. Generally, a plasmid vector is introduced in a precipitate, such as a calcium phosphate precipitate, or in a complex with a charged lipid. If the vector is a virus, it may be packaged in vitro using an appropriate packaging cell line and then transduced into host cells.

[0248] The stanniocalcin polynucleotide insert should be operatively linked to an appropriate promoter, such as the phage lambda PL promoter, the E. coli lac, trp, phoA and tac promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name a few. Other suitable promoters will be known to the skilled artisan. The expression constructs will further contain sites for transcription initiation, termination, and, in the transcribed region, a ribosome binding site for translation. The coding portion of the transcripts expressed by the constructs will preferably include a translation initiating codon at the beginning and a termination codon (UAA, UGA or UAG) appropriately positioned at the end of the polypeptide to be translated.

[0249] As indicated, the expression vectors will preferably include at least one selectable marker. Such markers include dihydrofolate reductase, G418 or neomycin resistance for eukaryotic cell culture and tetracycline, kanamycin or ampicillin resistance

genes for culturing in *E. coli* and other bacteria. Representative examples of appropriate hosts include, but are not limited to, bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast cells; insect cells such as *Drosophila* S2 and *Spodoptera Sf9* cells; animal cells such as CHO, COS, 293, and Bowes melanoma cells; and plant cells. Appropriate culture mediums and conditions for the above-described host cells are known in the art.

[0250] Among vectors preferred for use in bacteria include pQE70, pQE60 and pQE-9, available from QIAGEN, Inc.; pBluescript vectors, Phagescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene Cloning Systems, Inc.; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia Biotech, Inc. Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. Other suitable vectors will be readily apparent to the skilled artisan.

[0251] Vectors which use glutamine synthase (GS) or DHFR as the selectable markers can be amplified in the presence of the drugs methionine sulphoximine or methotrexate, respectively. An advantage of glutamine synthase based vectors are the availability of cell lines (e.g., the murine myeloma cell line, NS0) which are glutamine synthase negative. Glutamine synthase expression systems can also function in glutamine synthase expressing cells (e.g., Chinese Hamster Ovary (CHO) cells) by providing additional inhibitor to prevent the functioning of the endogenous gene. A glutamine synthase expression system and components thereof are detailed in PCT publications: WO87/04462; WO86/05807; WO89/01036; WO89/10404; and WO91/06657 which are hereby incorporated in their entireties by reference herein. Additionally, glutamine synthase expression vectors can be obtained from Lonza Biologics, Inc. (Portsmouth, NH). Expression and production of monoclonal antibodies using a GS expression system in murine myeloma cells is described in Bebbington *et al.*, *Bio/technology* 10:169(1992) and in Biblia and Robinson *Biotechnol. Prog.* 11:1 (1995) which are herein incorporated by reference.

[0252] The present invention also relates to host cells containing the above-described vector constructs described herein, and additionally encompasses host cells containing nucleotide sequences of the invention that are operably associated with one or more heterologous control regions (e.g., promoter and/or enhancer) using techniques known of in the art. The host cell can be a higher eukaryotic cell, such as a mammalian cell (e.g., a

human derived cell), or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. A host strain may be chosen which modulates the expression of the inserted gene sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus expression of the genetically engineered polypeptide may be controlled. Furthermore, different host cells have characteristics and specific mechanisms for the translational and post-translational processing and modification (e.g., phosphorylation, cleavage) of proteins. Appropriate cell lines can be chosen to ensure the desired modifications and processing of the foreign protein expressed.

[0253] Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection, or other methods. Such methods are described in many standard laboratory manuals, such as Davis et al., *Basic Methods In Molecular Biology* (1986). It is specifically contemplated that stanniocalcin polypeptides may in fact be expressed by a host cell lacking a recombinant vector.

[0254] Stanniocalcin polypeptides can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification.

[0255] Stanniocalcin polypeptides, and preferably the secreted form, can also be recovered from: products purified from natural sources, including bodily fluids, tissues and cells, whether directly isolated or cultured; products of chemical synthetic procedures; and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect, and mammalian cells. Depending upon the host employed in a recombinant production procedure, the stanniocalcin polypeptides may be glycosylated or may be non-glycosylated. In addition, stanniocalcin polypeptides may also include an initial modified methionine residue, in some cases as a result of host-mediated processes. Thus, it is well known in the art that the N-terminal methionine encoded by the translation initiation codon generally is removed with high efficiency from any protein after translation in all eukaryotic cells.

While the N-terminal methionine on most proteins also is efficiently removed in most prokaryotes, for some proteins, this prokaryotic removal process is inefficient, depending on the nature of the amino acid to which the N-terminal methionine is covalently linked.

[0256] In addition to encompassing host cells containing the vector constructs discussed herein, the invention also encompasses primary, secondary, and immortalized host cells of vertebrate origin, particularly mammalian origin, that have been engineered to delete or replace endogenous genetic material (e.g., stanniocalcin coding sequence), and/or to include genetic material (e.g., heterologous polynucleotide sequences) that is operably associated with stanniocalcin polynucleotides of the invention, and which activates, alters, and/or amplifies endogenous stanniocalcin polynucleotides. For example, techniques known in the art may be used to operably associate heterologous control regions (e.g., promoter and/or enhancer) and endogenous stanniocalcin polynucleotide sequences via homologous recombination (see, e.g., U.S. Patent No. 5,641,670; International Publication No. WO 96/29411; International Publication No. WO 94/12650; Koller *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:8932-8935 (1989); and Zijlstra *et al.*, *Nature*, 342:35-438 (1989), the disclosures of each of which are incorporated by reference in their entireties).

[0257] In addition, polypeptides of the invention can be chemically synthesized using techniques known in the art (e.g., see Creighton, 1983, *Proteins: Structures and Molecular Principles*, W.H. Freeman & Co., N.Y.; and Hunkapiller *et al.*, 1984, *Nature*, 310:105-111). For example, a peptide corresponding to a fragment of the stanniocalcin polypeptides of the invention can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the stanniocalcin polynucleotide sequence. Non-classical amino acids include, but are not limited to, to the D-isomers of the common amino acids, 2,4-diaminobutyric acid, α -amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, g-Abu, e-Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, b-alanine, fluoro-amino acids, designer amino acids such as b-methyl amino acids, Ca-methyl amino acids, Na-methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

[0258] The invention encompasses stanniocalcin polypeptides which are differentially modified during or after translation, e.g., by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications may be carried out by known techniques, including but not limited, to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH₄; acetylation, formylation, oxidation, reduction; metabolic synthesis in the presence of tunicamycin; etc.

[0259] Additional post-translational modifications encompassed by the invention include, for example, e.g., N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends), attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked or O-linked carbohydrate chains, and addition or deletion of an N-terminal methionine residue as a result of prokaryotic host cell expression. The polypeptides may also be modified with a detectable label, such as an enzymatic, fluorescent, isotopic or affinity label to allow for detection and isolation of the protein.

[0260] Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin; and examples of suitable radioactive material include iodine (¹²¹I, ¹²³I, ¹²⁵I, ¹³¹I), carbon (¹⁴C), sulfur (³⁵S), tritium (³H), indium (¹¹¹In, ¹¹²In, ^{113m}In, ^{115m}In), technetium (⁹⁹Tc, ^{99m}Tc), thallium (²⁰¹Tl), gallium (⁶⁸Ga, ⁶⁷Ga), palladium (¹⁰³Pd), molybdenum (⁹⁹Mo), xenon (¹³³Xe), fluorine (¹⁸F), ¹⁵³Sm, ¹⁷⁷Lu, ¹⁵⁹Gd, ¹⁴⁹Pm, ¹⁴⁰La, ¹⁷⁵Yb, ¹⁶⁶Ho, ⁹⁰Y, ⁴⁷Sc, ¹⁸⁶Re, ¹⁸⁸Re, ¹⁴²Pr, ¹⁰⁵Rh, and ⁹⁷Ru.

[0261] In specific embodiments, a polypeptide of the present invention or fragment or variant thereof is attached to macrocyclic chelators that associate with radiometal ions, including but not limited to, ¹⁷⁷Lu, ⁹⁰Y, ¹⁶⁶Ho, and ¹⁵³Sm, to polypeptides. In a preferred embodiment, the radiometal ion associated with the macrocyclic chelators is ¹¹¹In. In another preferred embodiment, the radiometal ion associated with the macrocyclic chelator

is ^{90}Y . In specific embodiments, the macrocyclic chelator is 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (DOTA). In other specific embodiments, DOTA is attached to an antibody of the invention or fragment thereof via a linker molecule. Examples of linker molecules useful for conjugating DOTA to a polypeptide are commonly known in the art - see, for example, DeNardo et al., Clin Cancer Res. 4(10):2483-90 (1998); Peterson et al., Bioconjug. Chem. 10(4):553-7 (1999); and Zimmerman et al, Nucl. Med. Biol. 26(8):943-50 (1999); which are hereby incorporated by reference in their entirety.

[0262] Also provided by the invention are chemically modified derivatives of stannioalcin which may provide additional advantages such as increased solubility, stability and circulating time of the polypeptide, or decreased immunogenicity (see U. S. Patent No. 4,179,337). The chemical moieties for derivitization may be selected from water soluble polymers such as polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, polyvinyl alcohol and the like. The polypeptides may be modified at random positions within the molecule, or at predetermined positions within the molecule and may include one, two, three or more attached chemical moieties.

[0263] The polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene glycol, the preferred molecular weight is between about 1 kDa and about 100 kDa (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight) for ease in handling and manufacturing. Other sizes may be used, depending on the desired therapeutic profile (e.g., the duration of sustained release desired, the effects, if any on biological activity, the ease in handling, the degree or lack of antigenicity and other known effects of the polyethylene glycol to a therapeutic protein or analog). For example, the polyethylene glycol may have an average molecular weight of about 200, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10,000, 10,500, 11,000, 11,500, 12,000, 12,500, 13,000, 13,500, 14,000, 14,500, 15,000, 15,500, 16,000, 16,500, 17,000, 17,500, 18,000, 18,500, 19,000, 19,500, 20,000, 25,000, 30,000, 35,000, 40,000, 45,000, 50,000, 55,000, 60,000, 65,000, 70,000, 75,000, 80,000, 85,000, 90,000, 95,000, or 100,000 kDa.

[0264] As noted above, the polyethylene glycol may have a branched structure. Branched polyethylene glycols are described, for example, in U.S. Patent No. 5,643,575; Morpurgo et al., *Appl. Biochem. Biotechnol.* 56:59-72 (1996); Vorobjev et al., *Nucleosides Nucleotides* 18:2745-2750 (1999); and Caliceti et al., *Bioconjug. Chem.* 10:638-646 (1999), the disclosures of each of which are incorporated herein by reference.

[0265] The polyethylene glycol molecules (or other chemical moieties) should be attached to the protein with consideration of effects on functional or antigenic domains of the protein. There are a number of attachment methods available to those skilled in the art, such as, for example, the method disclosed in EP 0 401 384 (coupling PEG to G-CSF), herein incorporated by reference; see also Malik et al., *Exp. Hematol.* 20:1028-1035 (1992), reporting pegylation of GM-CSF using tresyl chloride. For example, polyethylene glycol may be covalently bound through amino acid residues via a reactive group, such as a free amino or carboxyl group. Reactive groups are those to which an activated polyethylene glycol molecule may be bound. The amino acid residues having a free amino group may include lysine residues and the N-terminal amino acid residues; those having a free carboxyl group may include aspartic acid residues glutamic acid residues and the C-terminal amino acid residue. Sulfhydryl groups may also be used as a reactive group for attaching the polyethylene glycol molecules. Preferred for therapeutic purposes is attachment at an amino group, such as attachment at the N-terminus or lysine group.

[0266] As suggested above, polyethylene glycol may be attached to proteins via linkage to any of a number of amino acid residues. For example, polyethylene glycol can be linked to proteins via covalent bonds to lysine, histidine, aspartic acid, glutamic acid, or cysteine residues. One or more reaction chemistries may be employed to attach polyethylene glycol to specific amino acid residues (e.g., lysine, histidine, aspartic acid, glutamic acid, or cysteine) of the protein or to more than one type of amino acid residue (e.g., lysine, histidine, aspartic acid, glutamic acid, cysteine and combinations thereof) of the protein.

[0267] One may specifically desire proteins chemically modified at the N-terminus. Using polyethylene glycol as an illustration of the present composition, one may select from a variety of polyethylene glycol molecules (by molecular weight, branching, etc.), the proportion of polyethylene glycol molecules to protein (polypeptide) molecules in the reaction mix, the type of pegylation reaction to be performed, and the method of obtaining

the selected N-terminally pegylated protein. The method of obtaining the N-terminally pegylated preparation (i.e., separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of pegylated protein molecules. Selective proteins chemically modified at the N-terminus modification may be accomplished by reductive alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl group containing polymer is achieved.

[0268] As indicated above, pegylation of the proteins of the invention may be accomplished by any number of means. For example, polyethylene glycol may be attached to the protein either directly or by an intervening linker. Linkerless systems for attaching polyethylene glycol to proteins are described in Delgado et al., Crit. Rev. Thera. Drug Carrier Sys. 9:249-304 (1992); Francis et al., Intern. J. of Hematol. 68:1-18 (1998); U.S. Patent No. 4,002,531; U.S. Patent No. 5,349,052; WO 95/06058; and WO 98/32466, the disclosures of each of which are incorporated herein by reference.

[0269] One system for attaching polyethylene glycol directly to amino acid residues of proteins without an intervening linker employs tresylated MPEG, which is produced by the modification of monmethoxy polyethylene glycol (MPEG) using tresylchloride ($\text{ClSO}_2\text{CH}_2\text{CF}_3$). Upon reaction of protein with tresylated MPEG, polyethylene glycol is directly attached to amine groups of the protein. Thus, the invention includes protein-polyethylene glycol conjugates produced by reacting proteins of the invention with a polyethylene glycol molecule having a 2,2,2-trifluoroethane sulphonyl group.

[0270] Polyethylene glycol can also be attached to proteins using a number of different intervening linkers. For example, U.S. Patent No. 5,612,460, the entire disclosure of which is incorporated herein by reference, discloses urethane linkers for connecting polyethylene glycol to proteins. Protein-polyethylene glycol conjugates wherein the polyethylene glycol is attached to the protein by a linker can also be produced by reaction of proteins with compounds such as MPEG-succinimidylsuccinate, MPEG activated with 1,1'-carbonyldiimidazole, MPEG-2,4,5-trichloropenylcarbonate, MPEG-p-nitrophenolcarbonate, and various MPEG-succinate derivatives. A number of additional polyethylene glycol derivatives and reaction chemistries for attaching polyethylene glycol

to proteins are described in International Publication No. WO 98/32466, the entire disclosure of which is incorporated herein by reference. Pegylated protein products produced using the reaction chemistries set out herein are included within the scope of the invention.

[0271] The number of polyethylene glycol moieties attached to each protein of the invention (i.e., the degree of substitution) may also vary. For example, the pegylated proteins of the invention may be linked, on average, to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 17, 20, or more polyethylene glycol molecules. Similarly, the average degree of substitution within ranges such as 1-3, 2-4, 3-5, 4-6, 5-7, 6-8, 7-9, 8-10, 9-11, 10-12, 11-13, 12-14, 13-15, 14-16, 15-17, 16-18, 17-19, or 18-20 polyethylene glycol moieties per protein molecule. Methods for determining the degree of substitution are discussed, for example, in Delgado et al., *Crit. Rev. Thera. Drug Carrier Sys.* 9:249-304 (1992).

[0272] The stanniocalcin polypeptides of the invention can be recovered and purified from chemical synthesis and recombinant cell cultures by standard methods which include, but are not limited to, ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification. Well known techniques for refolding protein may be employed to regenerate active conformation when the polypeptide is denatured during isolation and/or purification.

[0273] The stanniocalcin polypeptides of the invention may be in monomers or multimers (i.e., dimers, trimers, tetramers and higher multimers). Accordingly, the present invention relates to monomers and multimers of the stanniocalcin polypeptides of the invention, their preparation, and compositions (preferably, pharmaceutical compositions) containing them. In specific embodiments, the polypeptides of the invention are monomers, dimers, trimers or tetramers. In additional embodiments, the multimers of the invention are at least dimers, at least trimers, or at least tetramers.

[0274] Multimers encompassed by the invention may be homomers or heteromers. As used herein, the term homomer, refers to a multimer containing only stanniocalcin polypeptides of the invention (including stanniocalcin fragments, variants, splice variants, and fusion proteins, as described herein). These homomers may contain stanniocalcin

polypeptides having identical or different amino acid sequences. In a specific embodiment, a homomer of the invention is a multimer containing only stanniocalcin polypeptides having an identical amino acid sequence. In another specific embodiment, a homomer of the invention is a multimer containing stanniocalcin polypeptides having different amino acid sequences. In specific embodiments, the multimer of the invention is a homodimer (e.g., containing stanniocalcin polypeptides having identical or different amino acid sequences) or a homotrimer (e.g., containing stanniocalcin polypeptides having identical and/or different amino acid sequences). In additional embodiments, the homomeric multimer of the invention is at least a homodimer, at least a homotrimer, or at least a homotetramer.

[0275] As used herein, the term heteromer refers to a multimer containing more than one heterologous polypeptides (i.e., polypeptides of different proteins) in addition to the stanniocalcin polypeptides of the invention. In a specific embodiment, the multimer of the invention is a heterodimer, a heterotrimer, or a heterotetramer. In additional embodiments, the heteromeric multimer of the invention is at least a heterodimer, at least a heterotrimer, or at least a heterotetramer.

[0276] Multimers of the invention may be the result of hydrophobic, hydrophilic, ionic and/or covalent associations and/or may be indirectly linked, by for example, liposome formation. Thus, in one embodiment, multimers of the invention, such as, for example, homodimers or homotrimers, are formed when polypeptides of the invention contact one another in solution. In another embodiment, heteromultimers of the invention, such as, for example, heterotrimers or heterotetramers, are formed when polypeptides of the invention contact antibodies to the polypeptides of the invention (including antibodies to the heterologous polypeptide sequence in a fusion protein of the invention) in solution. In other embodiments, multimers of the invention are formed by covalent associations with and/or between the stanniocalcin polypeptides of the invention. Such covalent associations may involve one or more amino acid residues contained in the polypeptide sequence (e.g., that recited in SEQ ID NO:2, or contained in the polypeptide encoded by the plasmid stanniocalcin). In one instance, the covalent associations are cross-linking between cysteine residues located within the polypeptide sequences which interact in the native (i.e., naturally occurring) polypeptide. In another instance, the covalent associations are the consequence of chemical or recombinant manipulation. Alternatively, such

covalent associations may involve one or more amino acid residues contained in the heterologous polypeptide sequence in a stanniocalcin fusion protein. In one example, covalent associations are between the heterologous sequence contained in a fusion protein of the invention (see, e.g., US Patent Number 5,478,925). In a specific example, the covalent associations are between the heterologous sequence contained in a stanniocalcin-Fc fusion protein of the invention (as described herein). In another specific example, covalent associations of fusion proteins of the invention are between heterologous polypeptide sequence from another protein that is capable of forming covalently associated multimers, such as for example, osteoprotegerin (see, e.g., International Publication No. WO 98/49305, the contents of which are herein incorporated by reference in its entirety).

[0277] The multimers of the invention may be generated using chemical techniques known in the art. For example, polypeptides desired to be contained in the multimers of the invention may be chemically cross-linked using linker molecules and linker molecule length optimization techniques known in the art (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). Additionally, multimers of the invention may be generated using techniques known in the art to form one or more inter-molecule cross-links between the cysteine residues located within the sequence of the polypeptides desired to be contained in the multimer (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). Further, polypeptides of the invention may be routinely modified by the addition of cysteine or biotin to the C terminus or N-terminus of the polypeptide and techniques known in the art may be applied to generate multimers containing one or more of these modified polypeptides (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). Additionally, techniques known in the art may be applied to generate liposomes containing the polypeptide components desired to be contained in the multimer of the invention (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety).

[0278] Alternatively, multimers of the invention may be generated using genetic engineering techniques known in the art. In one embodiment, polypeptides contained in multimers of the invention are produced recombinantly using fusion protein technology described herein or otherwise known in the art (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). In a specific embodiment,

polynucleotides coding for a homodimer of the invention are generated by ligating a polynucleotide sequence encoding a polypeptide of the invention to a sequence encoding a linker polypeptide and then further to a synthetic polynucleotide encoding the translated product of the polypeptide in the reverse orientation from the original C-terminus to the N-terminus (lacking the leader sequence) (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). In another embodiment, recombinant techniques described herein or otherwise known in the art are applied to generate recombinant polypeptides of the invention which contain a transmembrane domain (or hydrophobic or signal peptide) and which can be incorporated by membrane reconstitution techniques into liposomes (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety).

Uses of the Stanniocalcin Polynucleotides

[0279] The stanniocalcin polynucleotides identified herein can be used in numerous ways as reagents. The following description should be considered exemplary and utilizes known techniques. Further uses of Stanniocalcin polynucleotides are disclosed in International Publication No. WO 95/24411, which is herein incorporated by reference in its entirety.

[0280] There exists an ongoing need to identify new chromosome markers, since few chromosome marking reagents, based on actual sequence data (repeat polymorphisms), are presently available. The gene encoding the disclosed cDNA is thought to reside on chromosome 8. Accordingly, polynucleotides related to this invention are useful as a marker in linkage analysis for chromosome 8.

[0281] Briefly, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the sequences shown in SEQ ID NO:1. Primers can be selected using computer analysis so that primers do not span more than one predicted exon in the genomic DNA. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human stanniocalcin gene corresponding to the SEQ ID NO:1 will yield an amplified fragment.

[0282] Similarly, somatic hybrids provide a rapid method of PCR mapping the polynucleotides to particular chromosomes. Three or more plasmids can be assigned per

day using a single thermal cycler. Moreover, sublocalization of the stanniocalcin polynucleotides can be achieved with panels of specific chromosome fragments. Other gene mapping strategies that can be used include in situ hybridization, prescreening with labeled flow-sorted chromosomes, and preselection by hybridization to construct chromosome specific-cDNA libraries.

[0283] Precise chromosomal location of the stanniocalcin polynucleotides can also be achieved using fluorescence in situ hybridization (FISH) of a metaphase chromosomal spread. This technique uses polynucleotides as short as 500 or 600 bases; however, polynucleotides 2,000-4,000 bp are preferred. For a review of this technique, see Verma et al., "Human Chromosomes: a Manual of Basic Techniques," Pergamon Press, New York (1988).

[0284] For chromosome mapping, the stanniocalcin polynucleotides can be used individually (to mark a single chromosome or a single site on that chromosome) or in panels (for marking multiple sites and/or multiple chromosomes). Preferred polynucleotides correspond to the noncoding regions of the cDNAs because the coding sequences are more likely conserved within gene families, thus increasing the chance of cross hybridization during chromosomal mapping.

[0285] Once a polynucleotide has been mapped to a precise chromosomal location, the physical position of the polynucleotide can be used in linkage analysis. Linkage analysis establishes coinheritance between a chromosomal location and presentation of a particular disease. (Disease mapping data are found, for example, in V. McKusick, Mendelian Inheritance in Man (available on line through Johns Hopkins University Welch Medical Library) .) Assuming 1 megabase mapping resolution and one gene per 20 kb, a cDNA precisely localized to a chromosomal region associated with the disease could be one of 50-500 potential causative genes.

[0286] Thus, once coinheritance is established, differences in the stanniocalcin polynucleotide and the corresponding gene between affected and unaffected individuals can be examined. First, visible structural alterations in the chromosomes, such as deletions or translocations, are examined in chromosome spreads or by PCR. If no structural alterations exist, the presence of point mutations are ascertained. Mutations observed in some or all affected individuals, but not in normal individuals, indicates that the mutation may cause the disease. However, complete sequencing of the stanniocalcin

polypeptide and the corresponding gene from several normal individuals is required to distinguish the mutation from a polymorphism. If a new polymorphism is identified, this polymorphic polypeptide can be used for further linkage analysis.

[0287] Furthermore, increased or decreased expression of the gene in affected individuals as compared to unaffected individuals can be assessed using stanniocalcin polynucleotides. Any of these alterations (altered expression, chromosomal rearrangement, or mutation) can be used as a diagnostic or prognostic marker.

[0288] In addition to the foregoing, a stanniocalcin polynucleotide can be used to control gene expression through triple helix formation or antisense DNA or RNA. Both methods rely on binding of the polynucleotide to DNA or RNA. For these techniques, preferred polynucleotides are usually 20 to 40 bases in length and complementary to either the region of the gene involved in transcription (triple helix - see Lee *et al.*, *Nucl. Acids Res.*, 6:3073 (1979); Cooney *et al.*, *Science* 241:456 (1988); and Dervan *et al.*, *Science*, 251:1360 (1991)) or to the mRNA itself (antisense - Okano, *J. Neurochem.*, 56:560 (1991); Oligodeoxy-nucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988).) Triple helix formation optimally results in a shut-off of RNA transcription from DNA, while antisense RNA hybridization blocks translation of an mRNA molecule into polypeptide. Both techniques are effective in model systems, and the information disclosed herein can be used to design antisense or triple helix polynucleotides in an effort to treat disease.

[0289] Stanniocalcin polynucleotides are also useful in gene therapy. One goal of gene therapy is to insert a normal gene into an organism having a defective gene, in an effort to correct the genetic defect. Stanniocalcin offers a means of targeting such genetic defects in a highly accurate manner. Another goal is to insert a new gene that was not present in the host genome, thereby producing a new trait in the host cell.

[0290] The stanniocalcin polynucleotides are also useful for identifying individuals from minute biological samples. The United States military, for example, is considering the use of restriction fragment length polymorphism (RFLP) for identification of its personnel. In this technique, an individual's genomic DNA is digested with one or more restriction enzymes, and probed on a Southern blot to yield unique bands for identifying personnel. This method does not suffer from the current limitations of "Dog Tags" which

can be lost, switched, or stolen, making positive identification difficult. The stanniocalcin polynucleotides can be used as additional DNA markers for RFLP.

[0291] The stanniocalcin polynucleotides can also be used as an alternative to RFLP, by determining the actual base-by-base DNA sequence of selected portions of an individual's genome. These sequences can be used to prepare PCR primers for amplifying and isolating such selected DNA, which can then be sequenced. Using this technique, individuals can be identified because each individual will have a unique set of DNA sequences. Once an unique ID database is established for an individual, positive identification of that individual, living or dead, can be made from extremely small tissue samples.

[0292] Forensic biology also benefits from using DNA-based identification techniques as disclosed herein. DNA sequences taken from very small biological samples such as tissues, e.g., hair or skin, or body fluids, e.g., blood, saliva, semen, etc., can be amplified using PCR. In one prior art technique, gene sequences amplified from polymorphic loci, such as DQa class II HLA gene, are used in forensic biology to identify individuals. (Erlich, H., PCR Technology, Freeman and Co. (1992)). Once these specific polymorphic loci are amplified, they are digested with one or more restriction enzymes, yielding an identifying set of bands on a Southern blot probed with DNA corresponding to the DQa class II HLA gene. Similarly, stanniocalcin polynucleotides can be used as polymorphic markers for forensic purposes.

[0293] There is also a need for reagents capable of identifying the source of a particular tissue. Such need arises, for example, in forensics when presented with tissue of unknown origin. Appropriate reagents can comprise, for example, DNA probes or primers specific to particular tissue prepared from stanniocalcin sequences. Panels of such reagents can identify tissue by species and/or by organ type. In a similar fashion, these reagents can be used to screen tissue cultures for contamination.

[0294] Because stanniocalcin is found expressed in stromal cells from thymus and bone marrow, stanniocalcin polynucleotides are useful as hybridization probes for differential identification of the tissue(s) or cell type(s) present in a biological sample. Similarly, polypeptides and antibodies directed to stanniocalcin polypeptides are useful to provide immunological probes for differential identification of the tissue(s) or cell type(s). In addition, for a number of disorders of the above tissues or cells, particularly of the skeletal

and neural systems, significantly higher or lower levels of stanniocalcin gene expression may be detected in certain tissues (e.g., neural, skeletal, cancerous and wounded tissues) or bodily fluids (e.g., lymph, serum, plasma, urine, synovial fluid or spinal fluid) taken from an individual having such a disorder, relative to a “standard” stanniocalcin gene expression level, i.e., the stanniocalcin expression level in healthy tissue from an individual not having the stanniocalcin system disorder.

[0295] Thus, the invention provides a diagnostic method of a disorder, which involves: (a) assaying stanniocalcin gene expression level in cells or body fluid of an individual; (b) comparing the stanniocalcin gene expression level with a standard stanniocalcin gene expression level, whereby an increase or decrease in the assayed stanniocalcin gene expression level compared to the standard expression level is indicative of disorder in the stanniocalcin system.

[0296] In the very least, the stanniocalcin polynucleotides can be used as molecular weight markers on Southern gels, as diagnostic probes for the presence of a specific mRNA in a particular cell type, as a probe to “subtract-out” known sequences in the process of discovering novel polynucleotides, for selecting and making oligomers for attachment to a “gene chip” or other support, to raise anti-DNA antibodies using DNA immunization techniques, and as an antigen to elicit an immune response.

Uses of Stanniocalcin Polypeptides

[0297] Stanniocalcin polypeptides of the invention have numerous uses. The following description should be considered exemplary and utilizes known techniques. Further uses of Stanniocalcin polypeptides are disclosed in International Publication No. WO 95/24411, which is herein incorporated by reference in its entirety.

[0298] Altered levels of stanniocalcin protein in a biological sample relative to that in an average individual is likely to be indicative of neural injury and/or a propensity for neural injury. Accordingly, stanniocalcin polypeptides of the invention and antibodies generated against stanniocalcin polypeptides of the invention can be used in assays such as immunoassays to detect, prognose, diagnose or monitor neural injury, neural diseases or disorders, or to monitor the treatment thereof.

[0299] As discussed herein, stanniocalcin polypeptides of the invention have uses that include, but are not limited to, treating or protecting neural cells. In specific embodiments,

the stanniocalcin polypeptides of the invention are used to treat and/or prevent neural damage induced by hypoxia or ischemia (See Example 1).

[0300] Thus, in one embodiment, Stanniocalcin polypeptides are used to generate antibodies that can be used to assay stanniocalcin protein levels in a biological sample using antibody-based techniques. For example, protein expression in tissues can be studied with classical immunohistological methods. (Jalkanen *et al.*, *J. Cell. Biol.*, 101:976-985 (1985); Jalkanen *et al.*, *J. Cell. Biol.*, 105:3087-96 (1987)). Other antibody-based methods useful for detecting protein gene expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA). Suitable antibody assay labels are known in the art and include enzyme labels, such as, glucose oxidase, and radioisotopes, such as iodine (125I, 121I), carbon (14C), sulfur (35S), tritium (3H), indium (112In), and technetium (99mTc), and fluorescent labels, such as fluorescein and rhodamine, and biotin.

[0301] In addition to assaying secreted protein levels in a biological sample, proteins can also be detected in vivo by imaging. Antibody labels or markers for in vivo imaging of protein include those detectable by X-radiography, NMR or ESR. For X-radiography, suitable labels include radioisotopes such as barium or cesium, which emit detectable radiation but are not overtly harmful to the subject. Suitable markers for NMR and ESR include those with a detectable characteristic spin, such as deuterium, which may be incorporated into the antibody by labeling of nutrients for the relevant hybridoma.

[0302] A protein-specific antibody or antibody fragment which has been labeled with an appropriate detectable imaging moiety, such as a radioisotope (for example, 131I, 112In, 99mTc), a radio-opaque substance, or a material detectable by nuclear magnetic resonance, is introduced (for example, parenterally, subcutaneously, or intraperitoneally) into the mammal. It will be understood in the art that the size of the subject and the imaging system used will determine the quantity of imaging moiety needed to produce diagnostic images. In the case of a radioisotope moiety, for a human subject, the quantity of radioactivity injected will normally range from about 5 to 20 millicuries of 99mTc. The labeled antibody or antibody fragment will then preferentially accumulate at the location of cells which contain the specific protein. In vivo tumor imaging is described in S.W. Burchiel *et al.*, "Immunopharmacokinetics of Radiolabeled Antibodies and Their

Fragments.” (Chapter 13 in *Tumor Imaging: The Radiochemical Detection of Cancer*, S.W. Burchiel and B. A. Rhodes, eds., Masson Publishing Inc. (1982).)

[0303] Thus, the invention provides a method of detecting, prognosing, diagnosing, or monitoring neural injury and/or neural diseases or disorders or monitoring the treatment thereof. In particular, such an assay is carried out by a method comprising (a) assaying the expression of stanniocalcin polypeptide in cells or body fluid of an individual; (b) comparing the level of gene expression with a standard gene expression level, whereby an increase or decrease in the assayed stanniocalcin polypeptide gene expression level compared to the standard expression level is indicative of a neural injury and/or a neural disease or disorder and/or a predisposition for neural injury and/or a neural disease or disorder. In another embodiment, the assay is carried out by a method comprising (a) contacting a biological sample derived from an individual with an anti-stanniocalcin antibody under conditions such that immunospecific binding can occur; and (b) detecting or measuring the amount of any immunospecific binding by the antibody. In a specific embodiment, antibody to stanniocalcin can be used to assay in a biological sample for the presence of decreased levels of stanniocalcin. Decreased levels of endogenous stanniocalcin may be indicative of neural cell injury and/or a neural disease or disorder and/or a predisposition for neural injury and/or a neural disease or disorder. In a specific embodiment, antibody to stanniocalcin can be used to assay in a biological sample for the presence of increased levels of stanniocalcin. Increased levels of endogenous stanniocalcin may be indicative of neural cell injury and/or a neural disease or disorder and/or a predisposition for neural injury and/or a neural disease or disorder.

[0304] Moreover, stanniocalcin polypeptides can be used to treat disease. For example, patients can be administered stanniocalcin polypeptides in an effort to replace absent or decreased levels of the stanniocalcin polypeptide, to supplement absent or decreased levels of a different polypeptide, to inhibit the activity of a polypeptide, to activate the activity of a polypeptide, to reduce the activity of a membrane bound receptor by competing with it for free ligand, or to bring about a desired response.

[0305] Similarly, antibodies directed to stanniocalcin polypeptides can also be used to treat disease. For example, administration of an antibody directed to a stanniocalcin polypeptide can bind and reduce overproduction of the polypeptide. Similarly,

administration of an antibody can activate the polypeptide, such as by binding to a polypeptide bound to a membrane (receptor).

[0306] At the very least, the stanniocalcin polypeptides can be used as molecular weight markers on SDS-PAGE gels or on molecular sieve gel filtration columns using methods well known to those of skill in the art. Stanniocalcin polypeptides can also be used to raise antibodies, which in turn are used to measure protein expression from a recombinant cell, as a way of assessing transformation of the host cell. Moreover, stanniocalcin polypeptides can be used to test the following biological activities.

Gene Therapy Methods

[0307] Another aspect of the present invention is to gene therapy methods for treating disorders, diseases and conditions. The gene therapy methods relate to the introduction of nucleic acid (DNA, RNA and antisense DNA or RNA) sequences into an animal to achieve expression of the stanniocalcin polypeptide of the present invention. This method requires a polynucleotide which codes for a stanniocalcin polypeptide operatively linked to a promoter and any other genetic elements necessary for the expression of the polypeptide by the target tissue. Such gene therapy and delivery techniques are known in the art, see, for example, WO90/11092, which is herein incorporated by reference.

[0308] Thus, for example, cells from a patient may be engineered with a polynucleotide (DNA or RNA) comprising a promoter operably linked to a stanniocalcin polynucleotide *ex vivo*, with the engineered cells then being provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, see Beldegrun *et al.*, *J. Natl. Cancer Inst.*, 85: 207-16 (1993); Ferrantini *et al.*, *Cancer Research*, 53: 1107-12 (1993); Ferrantini *et al.*, *J. Immunology*, 153:4604-15 (1994); Kaido *et al.*, *Int. J. Cancer*, 60:221-29 (1995); Ogura *et al.*, *Cancer Research*, 50:5102-06 (1990); Santodonato *et al.*, *Human Gene Therapy*, 7:1-10 (1996); Santodonato *et al.*, *Gene Therapy*, 4:1246-1255 (1997); and Zhang *et al.*, *Cancer Gene Therapy*, 3:31-38 (1996)), which are herein incorporated by reference. In one embodiment, the cells which are engineered are arterial cells. The arterial cells may be reintroduced into the patient through direct injection to the artery, the tissues surrounding the artery, or through catheter injection.

[0309] As discussed in more detail below, the stanniocalcin polynucleotide constructs can be delivered by any method that delivers injectable materials to the cells of an animal,

such as, injection into the interstitial space of tissues (heart, muscle, skin, lung, liver, and the like). The stanniocalcin polynucleotide constructs may be delivered in a pharmaceutically acceptable liquid or aqueous carrier.

[0310] In one embodiment, the stanniocalcin polynucleotide is delivered as a naked polynucleotide. The term “naked” polynucleotide, DNA or RNA refers to sequences that are free from any delivery vehicle that acts to assist, promote or facilitate entry into the cell, including viral sequences, viral particles, liposome formulations, lipofectin or precipitating agents and the like. However, the stanniocalcin polynucleotides can also be delivered in liposome formulations and lipofectin formulations and the like can be prepared by methods well known to those skilled in the art. Such methods are described, for example, in U.S. Patent Nos. 5,593,972, 5,589,466, and 5,580,859, which are herein incorporated by reference.

[0311] The stanniocalcin polynucleotide vector constructs used in the gene therapy method are preferably constructs that will not integrate into the host genome nor will they contain sequences that allow for replication. Appropriate vectors include pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; pSVK3, pBPV, pMSG and pSVL available from Pharmacia; and pEF1/V5, pcDNA3.1, and pRc/CMV2 available from Invitrogen. Other suitable vectors will be readily apparent to the skilled artisan.

[0312] Any strong promoter known to those skilled in the art can be used for driving the expression of stanniocalcin DNA. Suitable promoters include adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoAI promoter; human globin promoters; viral thymidine kinase promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs; the b-actin promoter; and human growth hormone promoters. The promoter also may be the native promoter for stanniocalcin.

[0313] Unlike other gene therapy techniques, one major advantage of introducing naked nucleic acid sequences into target cells is the transitory nature of the polynucleotide synthesis in the cells. Studies have shown that non-replicating DNA sequences can be introduced into cells to provide production of the desired polypeptide for periods of up to six months.

[0314] The stanniocalcin polynucleotide construct can be delivered to the interstitial space of tissues within the an animal, including of muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, and connective tissue. Interstitial space of the tissues comprises the intercellular, fluid, mucopolysaccharide matrix among the reticular fibers of organ tissues, elastic fibers in the walls of vessels or chambers, collagen fibers of fibrous tissues, or that same matrix within connective tissue ensheathing muscle cells or in the lacunae of bone. It is similarly the space occupied by the plasma of the circulation and the lymph fluid of the lymphatic channels. Delivery to the interstitial space of muscle tissue is preferred for the reasons discussed below. They may be conveniently delivered by injection into the tissues comprising these cells. They are preferably delivered to and expressed in persistent, non-dividing cells which are differentiated, although delivery and expression may be achieved in non-differentiated or less completely differentiated cells, such as, for example, stem cells of blood or skin fibroblasts. In vivo muscle cells are particularly competent in their ability to take up and express polynucleotides.

[0315] For the naked nucleic acid sequence injection, an effective dosage amount of DNA or RNA will be in the range of from about 0.05 mg/kg body weight to about 50 mg/kg body weight. Preferably the dosage will be from about 0.005 mg/kg to about 20 mg/kg and more preferably from about 0.05 mg/kg to about 5 mg/kg. Of course, as the artisan of ordinary skill will appreciate, this dosage will vary according to the tissue site of injection. The appropriate and effective dosage of nucleic acid sequence can readily be determined by those of ordinary skill in the art and may depend on the condition being treated and the route of administration.

[0316] The preferred route of administration is by the parenteral route of injection into the interstitial space of tissues. However, other parenteral routes may also be used, such as, inhalation of an aerosol formulation particularly for delivery to lungs or bronchial tissues, throat or mucous membranes of the nose. In addition, naked stanniocalcin DNA constructs can be delivered to arteries during angioplasty by the catheter used in the procedure.

[0317] The naked polynucleotides are delivered by any method known in the art, including, but not limited to, direct needle injection at the delivery site, intravenous

injection, topical administration, catheter infusion, and so-called "gene guns". These delivery methods are known in the art.

[0318] The constructs may also be delivered with delivery vehicles such as viral sequences, viral particles, liposome formulations, lipofectin, precipitating agents, etc. Such methods of delivery are known in the art.

[0319] In certain embodiments, the stanniocalcin polynucleotide constructs are complexed in a liposome preparation. Liposomal preparations for use in the instant invention include cationic (positively charged), anionic (negatively charged) and neutral preparations. However, cationic liposomes are particularly preferred because a tight charge complex can be formed between the cationic liposome and the polyanionic nucleic acid. Cationic liposomes have been shown to mediate intracellular delivery of plasmid DNA (Felgner *et al.*, *Proc. Natl. Acad. Sci. USA*, 84:7413-16 (1987), which is herein incorporated by reference); mRNA (Malone *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:6077-6081 (1989), which is herein incorporated by reference); and purified transcription factors (Debs *et al.*, *J. Biol. Chem.* (1990) 265:10189-10192, which is herein incorporated by reference), in functional form.

[0320] Cationic liposomes are readily available. For example, N[1-2,3-dioleoyloxy)propyl]-N,N,N-triethylammonium (DOTMA) liposomes are particularly useful and are available under the trademark Lipofectin, from GIBCO BRL, Grand Island, N.Y. (See, also, Felgner *et al.*, *Proc. Natl. Acad. Sci. USA*, 84:7413-7416 (1987), which is herein incorporated by reference). Other commercially available liposomes include transfectace (DDAB/DOPE) and DOTAP/DOPE (Boehringer).

[0321] Other cationic liposomes can be prepared from readily available materials using techniques well known in the art. See, e.g. PCT Publication No. WO 90/11092 (which is herein incorporated by reference) for a description of the synthesis of DOTAP (1,2-bis(oleoyloxy)-3-(trimethylammonio)propane) liposomes. Preparation of DOTMA liposomes is explained in the literature (See, e.g., Felgner *et al.*, *Proc. Natl. Acad. Sci. USA*, 84:7413-7417 (1987), which is herein incorporated by reference). Similar methods can be used to prepare liposomes from other cationic lipid materials.

[0322] Similarly, anionic and neutral liposomes are readily available, such as from Avanti Polar Lipids (Birmingham, Ala.), or can be easily prepared using readily available materials. Such materials include phosphatidyl, choline, cholesterol, phosphatidyl

ethanolamine, dioleoylphosphatidyl choline (DOPC), dioleoylphosphatidyl glycerol (DOPG), dioleoylphosphatidyl ethanolamine (DOPE), among others. These materials can also be mixed with the DOTMA and DOTAP starting materials in appropriate ratios. Methods for making liposomes using these materials are well known in the art.

[0323] For example, commercially dioleoylphosphatidyl choline (DOPC), dioleoylphosphatidyl glycerol (DOPG), and dioleoylphosphatidyl ethanolamine (DOPE) can be used in various combinations to make conventional liposomes, with or without the addition of cholesterol. Thus, for example, DOPG/DOPC vesicles can be prepared by drying 50 mg each of DOPG and DOPC under a stream of nitrogen gas into a sonication vial. The sample is placed under a vacuum pump overnight and is hydrated the following day with deionized water. The sample is then sonicated for 2 hours in a capped vial, using a Heat Systems model 350 sonicator equipped with an inverted cup (bath type) probe at the maximum setting while the bath is circulated at 15EC. Alternatively, negatively charged vesicles can be prepared without sonication to produce multilamellar vesicles or by extrusion through nucleopore membranes to produce unilamellar vesicles of discrete size. Other methods are known and available to those of skill in the art.

[0324] The liposomes can comprise multilamellar vesicles (MLVs), small unilamellar vesicles (SUVs), or large unilamellar vesicles (LUVs), with SUVs being preferred. The various liposome-nucleic acid complexes are prepared using methods well known in the art. See, e.g., Straubinger et al., *Methods of Immunology* (1983), 101:512-527, which is herein incorporated by reference. For example, MLVs containing nucleic acid can be prepared by depositing a thin film of phospholipid on the walls of a glass tube and subsequently hydrating with a solution of the material to be encapsulated. SUVs are prepared by extended sonication of MLVs to produce a homogeneous population of unilamellar liposomes. The material to be entrapped is added to a suspension of preformed MLVs and then sonicated. When using liposomes containing cationic lipids, the dried lipid film is resuspended in an appropriate solution such as sterile water or an isotonic buffer solution such as 10 mM Tris/NaCl, sonicated, and then the preformed liposomes are mixed directly with the DNA. The liposome and DNA form a very stable complex due to binding of the positively charged liposomes to the cationic DNA. SUVs find use with small nucleic acid fragments. LUVs are prepared by a number of methods, well known in the art. Commonly used methods include Ca^{2+} -EDTA chelation (Papahadjopoulos *et al.*,

Biochim. Biophys. Acta, 394:483 (1975); Wilson *et al.*, *Cell*, 17:77 (1979)); ether injection (Deamer, D. and Bangham, A., *Biochim. Biophys. Acta*, 443:629 (1976); Ostro *et al.*, *Biochem. Biophys. Res. Commun.*, 76:836 (1977); Fraley *et al.*, *Proc. Natl. Acad. Sci. USA*, 76:3348 (1979)); detergent dialysis (Enoch, H. and Strittmatter, P., *Proc. Natl. Acad. Sci. USA*, 76:145 (1979)); and reverse-phase evaporation (REV) (Fraley *et al.*, *J. Biol. Chem.*, 255:10431 (1980); Szoka, F. and Papahadjopoulos, D., *Proc. Natl. Acad. Sci. USA*, 75:145 (1978); Schaefer-Ridder *et al.*, *Science*, 215:166 (1982)), which are herein incorporated by reference.

[0325] Generally, the ratio of DNA to liposomes will be from about 10:1 to about 1:10. Preferably, the ration will be from about 5:1 to about 1:5. More preferably, the ration will be about 3:1 to about 1:3. Still more preferably, the ratio will be about 1:1.

[0326] U.S. Patent No. 5,676,954 (which is herein incorporated by reference) reports on the injection of genetic material, complexed with cationic liposomes carriers, into mice. U.S. Patent Nos. 4,897,355, 4,946,787, 5,049,386, 5,459,127, 5,589,466, 5,693,622, 5,580,859, 5,703,055, and international publication no. WO 94/9469 (which are herein incorporated by reference) provide cationic lipids for use in transfecting DNA into cells and mammals. U.S. Patent Nos. 5,589,466, 5,693,622, 5,580,859, 5,703,055, and international publication no. WO 94/9469 (which are herein incorporated by reference) provide methods for delivering DNA-cationic lipid complexes to mammals.

[0327] In certain embodiments, cells are engineered, *ex vivo* or *in vivo*, using a retroviral particle containing RNA which comprises a sequence encoding stanniocalcin. Retroviruses from which the retroviral plasmid vectors may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, Rous sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, Myeloproliferative Sarcoma Virus, and mammary tumor virus.

[0328] The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317, R-2, R-AM, PA12, T19-14X, VT-19-17-H2, RCRE, RCRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, *Human Gene Therapy* 1:5-14 (1990), which is incorporated herein by reference in its entirety. The vector may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and

CaPO₄ precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host.

[0329] The producer cell line generates infectious retroviral vector particles which include polynucleotide encoding stanniocalcin. Such retroviral vector particles then may be employed, to transduce eukaryotic cells, either in vitro or in vivo. The transduced eukaryotic cells will express stanniocalcin.

[0330] In certain other embodiments, cells are engineered, ex vivo or in vivo, with stanniocalcin polynucleotide contained in an adenovirus vector. Adenovirus can be manipulated such that it encodes and expresses stanniocalcin, and at the same time is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. Adenovirus expression is achieved without integration of the viral DNA into the host cell chromosome, thereby alleviating concerns about insertional mutagenesis. Furthermore, adenoviruses have been used as live enteric vaccines for many years with an excellent safety profile (Schwartz *et al.*, *Am. Rev. Respir. Dis.*, 109:233-238 (1974)). Finally, adenovirus mediated gene transfer has been demonstrated in a number of instances including transfer of alpha-1-antitrypsin and CFTR to the lungs of cotton rats (Rosenfeld *et al.*, *Science*, 252:431-434 (1991); Rosenfeld *et al.*, *Cell*, 68:143-155 (1992)). Furthermore, extensive studies to attempt to establish adenovirus as a causative agent in human cancer were uniformly negative (Green *et al.*, *Proc. Natl. Acad. Sci. USA*, 76:6606 (1979)).

[0331] Suitable adenoviral vectors useful in the present invention are described, for example, in Kozarsky and Wilson, *Curr. Opin. Genet. Devel.*, 3:499-503 (1993); Rosenfeld *et al.*, *Cell*, 68:143-155 (1992); Engelhardt *et al.*, *Human Genet. Ther.*, 4:759-769 (1993); Yang *et al.*, *Nature Genet.*, 7:362-369 (1994); Wilson *et al.*, *Nature*, 365:691-692 (1993); and U.S. Patent No. 5,652,224, which are herein incorporated by reference. For example, the adenovirus vector Ad2 is useful and can be grown in human 293 cells. These cells contain the E1 region of adenovirus and constitutively express Ela and Elb, which complement the defective adenoviruses by providing the products of the genes deleted from the vector. In addition to Ad2, other varieties of adenovirus (e.g., Ad3, Ad5, and Ad7) are also useful in the present invention.

[0332] Preferably, the adenoviruses used in the present invention are replication deficient. Replication deficient adenoviruses require the aid of a helper virus and/or

packaging cell line to form infectious particles. The resulting virus is capable of infecting cells and can express a polynucleotide of interest which is operably linked to a promoter, but cannot replicate in most cells. Replication deficient adenoviruses may be deleted in one or more of all or a portion of the following genes: E1a, E1b, E3, E4, E2a, or L1 through L5.

[0333] In certain other embodiments, the cells are engineered, *ex vivo* or *in vivo*, using an adeno-associated virus (AAV). AAVs are naturally occurring defective viruses that require helper viruses to produce infectious particles (Muzyczka, N., *Curr. Topics in Microbiol. Immunol.* 158:97 (1992)). It is also one of the few viruses that may integrate its DNA into non-dividing cells. Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate, but space for exogenous DNA is limited to about 4.5 kb. Methods for producing and using such AAVs are known in the art. See, for example, U.S. Patent Nos. 5,139,941, 5,173,414, 5,354,678, 5,436,146, 5,474,935, 5,478,745, and 5,589,377.

[0334] For example, an appropriate AAV vector for use in the present invention will include all the sequences necessary for DNA replication, encapsidation, and host-cell integration. The stanniocalcin polynucleotide construct is inserted into the AAV vector using standard cloning methods, such as those found in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press (1989). The recombinant AAV vector is then transfected into packaging cells which are infected with a helper virus, using any standard technique, including lipofection, electroporation, calcium phosphate precipitation, etc. Appropriate helper viruses include adenoviruses, cytomegaloviruses, vaccinia viruses, or herpes viruses. Once the packaging cells are transfected and infected, they will produce infectious AAV viral particles which contain the stanniocalcin polynucleotide construct. These viral particles are then used to transduce eukaryotic cells, either *ex vivo* or *in vivo*. The transduced cells will contain the stanniocalcin polynucleotide construct integrated into its genome, and will express stanniocalcin.

[0335] Another method of gene therapy involves operably associating heterologous control regions and endogenous polynucleotide sequences (e.g. encoding stanniocalcin) via homologous recombination (see, e.g., U.S. Patent No. 5,641,670; International Publication No. WO 96/29411; International Publication No. WO 94/12650; Koller et al., *Proc. Natl. Acad. Sci. USA* 86:8932-8935 (1989); and Zijlstra et al., *Nature* 342:435-438

(1989). This method involves the activation of a gene which is present in the target cells, but which is not normally expressed in the cells, or is expressed at a lower level than desired.

[0336] Polynucleotide constructs are made, using standard techniques known in the art, which contain the promoter with targeting sequences flanking the promoter. Suitable promoters are described herein. The targeting sequence is sufficiently complementary to an endogenous sequence to permit homologous recombination of the promoter-targeting sequence with the endogenous sequence. The targeting sequence will be sufficiently near the 5' end of the stanniocalcin desired endogenous polynucleotide sequence so the promoter will be operably linked to the endogenous sequence upon homologous recombination.

[0337] The promoter and the targeting sequences can be amplified using PCR. Preferably, the amplified promoter contains distinct restriction enzyme sites on the 5' and 3' ends. Preferably, the 3' end of the first targeting sequence contains the same restriction enzyme site as the 5' end of the amplified promoter and the 5' end of the second targeting sequence contains the same restriction site as the 3' end of the amplified promoter. The amplified promoter and targeting sequences are digested and ligated together.

[0338] The promoter-targeting sequence construct is delivered to the cells, either as naked polynucleotide, or in conjunction with transfection-facilitating agents, such as liposomes, viral sequences, viral particles, whole viruses, lipofection, precipitating agents, etc., described in more detail above. The P promoter-targeting sequence can be delivered by any method, included direct needle injection, intravenous injection, topical administration, catheter infusion, particle accelerators, etc. The methods are described in more detail below.

[0339] The promoter-targeting sequence construct is taken up by cells. Homologous recombination between the construct and the endogenous sequence takes place, such that an endogenous stanniocalcin sequence is placed under the control of the promoter. The promoter then drives the expression of the endogenous stanniocalcin sequence.

[0340] Preferably, the polynucleotide encoding stanniocalcin contains a secretory signal sequence that facilitates secretion of the protein. Typically, the signal sequence is positioned in the coding region of the polynucleotide to be expressed towards or at the 5' end of the coding region. The signal sequence may be homologous or heterologous to the

polynucleotide of interest and may be homologous or heterologous to the cells to be transfected. Additionally, the signal sequence may be chemically synthesized using methods known in the art.

[0341] Any mode of administration of any of the above-described polynucleotides constructs can be used so long as the mode results in the expression of one or more molecules in an amount sufficient to provide a therapeutic effect. This includes direct needle injection, systemic injection, catheter infusion, biolistic injectors, particle accelerators (i.e., “gene guns”), gelfoam sponge depots, other commercially available depot materials, osmotic pumps (e.g., Alza minipumps), oral or suppository solid (tablet or pill) pharmaceutical formulations, and decanting or topical applications during surgery. For example, direct injection of naked calcium phosphate-precipitated plasmid into rat liver and rat spleen or a protein-coated plasmid into the portal vein has resulted in gene expression of the foreign gene in the rat livers (Kaneda *et al.*, *Science*, 243:375 (1989)).

[0342] A preferred method of local administration is by direct injection. Preferably, a recombinant molecule of the present invention complexed with a delivery vehicle is administered by direct injection into or locally within the area of arteries. Administration of a composition locally within the area of arteries refers to injecting the composition centimeters and preferably, millimeters within arteries.

[0343] Another method of local administration is to contact a polynucleotide construct of the present invention in or around a surgical wound. For example, a patient can undergo surgery and the polynucleotide construct can be coated on the surface of tissue inside the wound or the construct can be injected into areas of tissue inside the wound.

[0344] Therapeutic compositions useful in systemic administration, include recombinant molecules of the present invention complexed to a targeted delivery vehicle of the present invention. Suitable delivery vehicles for use with systemic administration comprise liposomes comprising ligands for targeting the vehicle to a particular site.

[0345] Preferred methods of systemic administration, include intravenous injection, aerosol, oral and percutaneous (topical) delivery. Intravenous injections can be performed using methods standard in the art. Aerosol delivery can also be performed using methods standard in the art (See, for example, Stribling *et al.*, *Proc. Natl. Acad. Sci. USA*, 189:11277-81 (1992), which is incorporated herein by reference). Oral delivery can be performed by complexing a polynucleotide construct of the present invention to a carrier

capable of withstanding degradation by digestive enzymes in the gut of an animal. Examples of such carriers, include plastic capsules or tablets, such as those known in the art. Topical delivery can be performed by mixing a polynucleotide construct of the present invention with a lipophilic reagent (e.g., DMSO) that is capable of passing into the skin.

[0346] Determining an effective amount of substance to be delivered can depend upon a number of factors including, for example, the chemical structure and biological activity of the substance, the age and weight of the animal, the precise condition requiring treatment and its severity, and the route of administration. The frequency of treatments depends upon a number of factors, such as the amount of polynucleotide constructs administered per dose, as well as the health and history of the subject. The precise amount, number of doses, and timing of doses will be determined by the attending physician or veterinarian.

[0347] Therapeutic compositions of the present invention can be administered to any animal, preferably to mammals and birds. Preferred mammals include humans, dogs, cats, mice, rats, rabbits sheep, cattle, horses and pigs, with humans being particularly preferred.

Biological Activities of Stanniocalcin

[0348] Stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, can be used in assays to test for one or more biological activities. If stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, do exhibit activity in a particular assay, it is likely that stanniocalcin may be involved in the diseases associated with the biological activity. Therefore, stanniocalcin could be used to diagnose, prognose, prevent and/or treat the associated disease.

[0349] Stanniocalcin polypeptides are believed to be involved in biological activities associated with the neural system, and in particular the protection of neural cells from the damaging effects of a hypoxic environment. Accordingly, compositions of the invention (including polynucleotides, polypeptides and antibodies of the invention, and fragments and variants thereof) may be used in the diagnosis, prognosis, prevention, and/or treatment of diseases and/or disorders associated with hypoxia and neural damage, as might result, for example, from stroke, heart attacks, and embolisms.

[0350] In preferred embodiments, compositions of the invention (including polynucleotides, polypeptides and antibodies of the invention, and fragments and variants thereof) may be used in the diagnosis, prognosis, prevention, and/or treatment of diseases

and/or disorders relating to neural disorders (e.g., neural damage from hypoxia, degenerative disorders, and/or as described under “Neural activity” below).

[0351] In certain embodiments, a polypeptide of the invention, or polynucleotides, antibodies, agonists, or antagonists corresponding to that polypeptide, may be used to diagnose and/or prognose diseases and/or disorders associated with the tissue(s) in which the Stanniocalcin polypeptides of the invention are expressed.

[0352] Thus, polynucleotides, translation products and antibodies of the invention are useful in the diagnosis, prognosis, prevention, and/or treatment of diseases and/or disorders associated with activities that include, but are not limited to, the protection of neural cells from the damaging effects of hypoxia and neural damage, as might result, for example, from stroke, heart attacks, and embolisms.

[0353] More generally, polynucleotides, translation products and antibodies corresponding to this gene may be useful for the diagnosis, prognosis, prevention, and/or treatment of diseases and/or disorders associated with the following systems.

Neural Activity

[0354] As disclosed herein, stanniocalcin compositions of the invention protect neural cells from damage and injury (see Example 1). Accordingly, the stanniocalcin polynucleotides, polypeptides and agonists or antagonists of the invention may be used for the diagnosis and/or treatment of diseases, disorders, damage or injury of the brain and/or nervous system. Nervous system disorders that can be treated with the compositions of the invention (e.g., polypeptides, polynucleotides, and/or agonists or antagonists), include, but are not limited to, nervous system injuries, and diseases or disorders which result in either a disconnection of axons, a diminution or degeneration of neurons, or demyelination. Nervous system lesions which may be treated in a patient (including human and non-human mammalian patients) according to the methods of the invention, include but are not limited to, the following lesions of either the central (including spinal cord, brain) or peripheral nervous systems: (1) ischemic lesions, in which a lack of oxygen in a portion of the nervous system results in neuronal injury or death, including cerebral infarction or ischemia, or spinal cord infarction or ischemia; (2) traumatic lesions, including lesions caused by physical injury or associated with surgery, for example, lesions which sever a portion of the nervous system, or compression injuries; (3) malignant lesions, in which a

portion of the nervous system is destroyed or injured by malignant tissue which is either a nervous system associated malignancy or a malignancy derived from non-nervous system tissue; (4) infectious lesions, in which a portion of the nervous system is destroyed or injured as a result of infection, for example, by an abscess or associated with infection by human immunodeficiency virus, herpes zoster, or herpes simplex virus or with Lyme disease, tuberculosis, or syphilis; (5) degenerative lesions, in which a portion of the nervous system is destroyed or injured as a result of a degenerative process including but not limited to, degeneration associated with Parkinson's disease, Alzheimer's disease, Huntington's chorea, or amyotrophic lateral sclerosis (ALS); (6) lesions associated with nutritional diseases or disorders, in which a portion of the nervous system is destroyed or injured by a nutritional disorder or disorder of metabolism including, but not limited to, vitamin B12 deficiency, folic acid deficiency, Wernicke disease, tobacco-alcohol amblyopia, Marchiafava-Bignami disease (primary degeneration of the corpus callosum), and alcoholic cerebellar degeneration; (7) neurological lesions associated with systemic diseases including, but not limited to, diabetes (diabetic neuropathy, Bell's palsy), systemic lupus erythematosus, carcinoma, or sarcoidosis; (8) lesions caused by toxic substances including alcohol, lead, or particular neurotoxins; and (9) demyelinated lesions in which a portion of the nervous system is destroyed or injured by a demyelinating disease including, but not limited to, multiple sclerosis, human immunodeficiency virus-associated myelopathy, transverse myelopathy or various etiologies, progressive multifocal leukoencephalopathy, and central pontine myelinolysis.

[0355] In one embodiment, the polypeptides, polynucleotides, or agonists or antagonists of the invention are used to protect neural cells from the damaging effects of hypoxia. In a further preferred embodiment, the polypeptides, polynucleotides, or agonists or antagonists of the invention are used to protect neural cells from the damaging effects of cerebral hypoxia. According to this embodiment, the compositions of the invention are used to treat or prevent neural cell injury associated with cerebral hypoxia. In one non-exclusive aspect of this embodiment, the polypeptides, polynucleotides, or agonists or antagonists of the invention, are used to treat or prevent neural cell injury associated with cerebral ischemia. In another non-exclusive aspect of this embodiment, the polypeptides, polynucleotides, or agonists or antagonists of the invention are used to treat or prevent neural cell injury associated with cerebral infarction.

[0356] In another preferred embodiment, the polypeptides, polynucleotides, or agonists or antagonists of the invention are used to treat or prevent neural cell injury associated with a stroke. In a specific embodiment, the polypeptides, polynucleotides, or agonists or antagonists of the invention are used to treat or prevent cerebral neural cell injury associated with a stroke.

[0357] In another preferred embodiment, the polypeptides, polynucleotides, or agonists or antagonists of the invention are used to treat or prevent neural cell injury associated with a heart attack. In a specific embodiment, the polypeptides, polynucleotides, or agonists or antagonists of the invention are used to treat or prevent cerebral neural cell injury associated with a heart attack.

[0358] The compositions of the invention which are useful for treating or preventing a nervous system disorder may be selected by testing for biological activity in promoting the survival or differentiation of neurons. For example, and not by way of limitation, compositions of the invention which elicit any of the following effects may be useful according to the invention: (1) increased survival time of neurons in culture either in the presence or absence of hypoxia or hypoxic conditions; (2) increased sprouting of neurons in culture or *in vivo*; (3) increased production of a neuron-associated molecule in culture or *in vivo*, e.g., choline acetyltransferase or acetylcholinesterase with respect to motor neurons; or (4) decreased symptoms of neuron dysfunction *in vivo*. Such effects may be measured by any method known in the art. In preferred, non-limiting embodiments, increased survival of neurons may routinely be measured using a method set forth herein or otherwise known in the art, such as, for example, in Zhang *et al.*, *Proc Natl Acad Sci USA* 97:3637-42 (2000) or in Arakawa *et al.*, *J. Neurosci.*, 10:3507-15 (1990); increased sprouting of neurons may be detected by methods known in the art, such as, for example, the methods set forth in Pestronk *et al.*, *Exp. Neurol.*, 70:65-82 (1980), or Brown *et al.*, *Ann. Rev. Neurosci.*, 4:17-42 (1981); increased production of neuron-associated molecules may be measured by bioassay, enzymatic assay, antibody binding, Northern blot assay, etc., using techniques known in the art and depending on the molecule to be measured; and motor neuron dysfunction may be measured by assessing the physical manifestation of motor neuron disorder, e.g., weakness, motor neuron conduction velocity, or functional disability.

[0359] In specific embodiments, motor neuron disorders that may be treated according to the invention include, but are not limited to, disorders such as infarction, infection, exposure to toxin, trauma, surgical damage, degenerative disease or malignancy that may affect motor neurons as well as other components of the nervous system, as well as disorders that selectively affect neurons such as amyotrophic lateral sclerosis, and including, but not limited to, progressive spinal muscular atrophy, progressive bulbar palsy, primary lateral sclerosis, infantile and juvenile muscular atrophy, progressive bulbar paralysis of childhood (Fazio-Londe syndrome), poliomyelitis and the post polio syndrome, and Hereditary Motorsensory Neuropathy (Charcot-Marie-Tooth Disease).

[0360] Further, polypeptides or polynucleotides of the invention may play a role in neuronal survival; synapse formation; conductance; neural differentiation, etc. Thus, compositions of the invention (including polynucleotides, polypeptides, and agonists or antagonists) may be used to diagnose and/or treat or prevent diseases or disorders associated with these roles, including, but not limited to, learning and/or cognition disorders. The compositions of the invention may also be useful in the treatment or prevention of neurodegenerative disease states and/or behavioral disorders. Such neurodegenerative disease states and/or behavioral disorders include, but are not limited to, Alzheimer's Disease, Parkinson's Disease, Huntington's Disease, Tourette Syndrome, schizophrenia, mania, dementia, paranoia, obsessive compulsive disorder, panic disorder, learning disabilities, ALS, psychoses, autism, and altered behaviors, including disorders in feeding, sleep patterns, balance, and perception. In addition, compositions of the invention may also play a role in the treatment, prevention and/or detection of developmental disorders associated with the developing embryo, or sexually-linked disorders.

[0361] Additionally, polypeptides, polynucleotides and/or agonists or antagonists of the invention, may be useful in protecting neural cells from diseases, damage, disorders, or injury, associated with cerebrovascular disorders including, but not limited to, carotid artery diseases (e.g., carotid artery thrombosis, carotid stenosis, or Moyamoya Disease), cerebral amyloid angiopathy, cerebral aneurysm, cerebral anoxia, cerebral arteriosclerosis, cerebral arteriovenous malformations, cerebral artery diseases, cerebral embolism and thrombosis (e.g., carotid artery thrombosis, sinus thrombosis, or Wallenberg's Syndrome), cerebral hemorrhage (e.g., epidural or subdural hematoma, or subarachnoid hemorrhage), cerebral infarction, cerebral ischemia (e.g., transient cerebral ischemia, Subclavian Steal

Syndrome, or vertebrobasilar insufficiency), vascular dementia (e.g., multi-infarct), leukomalacia, periventricular, and vascular headache (e.g., cluster headache or migraines).

[0362] In accordance with yet a further aspect of the present invention, there is provided a process for utilizing polynucleotides or polypeptides, as well as agonists or antagonists of the present invention, for therapeutic purposes, for example, to stimulate neurological cell proliferation and/or differentiation. Therefore, polynucleotides, polypeptides, agonists and/or antagonists of the invention may be used to treat and/or detect neurologic diseases. Moreover, polynucleotides or polypeptides, or agonists or antagonists of the invention, can be used as a marker or detector of a particular nervous system disease or disorder.

[0363] Examples of neurologic diseases which can be treated or detected with polynucleotides, polypeptides, agonists, and/or antagonists of the present invention include brain diseases, such as metabolic brain diseases which includes phenylketonuria such as maternal phenylketonuria, pyruvate carboxylase deficiency, pyruvate dehydrogenase complex deficiency, Wernicke's Encephalopathy, brain edema, brain neoplasms such as cerebellar neoplasms which include infratentorial neoplasms, cerebral ventricle neoplasms such as choroid plexus neoplasms, hypothalamic neoplasms, supratentorial neoplasms, canavan disease, cerebellar diseases such as cerebellar ataxia which include spinocerebellar degeneration such as ataxia telangiectasia, cerebellar dyssynergia, Friederich's Ataxia, Machado-Joseph Disease, olivopontocerebellar atrophy, cerebellar neoplasms such as infratentorial neoplasms, diffuse cerebral sclerosis such as encephalitis periaxialis, globoid cell leukodystrophy, metachromatic leukodystrophy and subacute sclerosing panencephalitis.

[0364] Additional neurologic diseases which can be treated or detected with polynucleotides, polypeptides, agonists, and/or antagonists of the present invention include cerebrovascular disorders (such as carotid artery diseases which include carotid artery thrombosis, carotid stenosis and Moyamoya Disease), cerebral amyloid angiopathy, cerebral aneurysm, cerebral anoxia, cerebral arteriosclerosis, cerebral arteriovenous malformations, cerebral artery diseases, cerebral embolism and thrombosis such as carotid artery thrombosis, sinus thrombosis and Wallenberg's Syndrome, cerebral hemorrhage such as epidural hematoma, subdural hematoma and subarachnoid hemorrhage, cerebral infarction, cerebral ischemia such as transient cerebral ischemia, Subclavian Steal Syndrome and vertebrobasilar insufficiency, vascular dementia such as multi-infarct

dementia, periventricular leukomalacia, vascular headache such as cluster headache and migraine.

[0365] Additional neurologic diseases which can be treated or detected with polynucleotides, polypeptides, agonists, and/or antagonists of the present invention include dementia such as AIDS Dementia Complex, presenile dementia such as Alzheimer's Disease and Creutzfeldt-Jakob Syndrome, senile dementia such as Alzheimer's Disease and progressive supranuclear palsy, vascular dementia such as multi-infarct dementia, encephalitis which include encephalitis periaxialis, viral encephalitis such as epidemic encephalitis, Japanese Encephalitis, St. Louis Encephalitis, tick-borne encephalitis and West Nile Fever, acute disseminated encephalomyelitis, meningoencephalitis such as uveomeningoencephalitic syndrome, Postencephalitic Parkinson Disease and subacute sclerosing panencephalitis, encephalomalacia such as periventricular leukomalacia, epilepsy such as generalized epilepsy which includes infantile spasms, absence epilepsy, myoclonic epilepsy which includes MERRF Syndrome, tonic-clonic epilepsy, partial epilepsy such as complex partial epilepsy, frontal lobe epilepsy and temporal lobe epilepsy, post-traumatic epilepsy, status epilepticus such as Epilepsia Partialis Continua, and Hallervorden-Spatz Syndrome.

[0366] Additional neurologic diseases which can be treated or detected with polynucleotides, polypeptides, agonists, and/or antagonists of the present invention include hydrocephalus such as Dandy-Walker Syndrome and normal pressure hydrocephalus, hypothalamic diseases such as hypothalamic neoplasms, cerebral malaria, narcolepsy which includes cataplexy, bulbar poliomyelitis, cerebri pseudotumor, Rett Syndrome, Reye's Syndrome, thalamic diseases, cerebral toxoplasmosis, intracranial tuberculoma and Zellweger Syndrome, central nervous system infections such as AIDS Dementia Complex, Brain Abscess, subdural empyema, encephalomyelitis such as Equine Encephalomyelitis, Venezuelan Equine Encephalomyelitis, Necrotizing Hemorrhagic Encephalomyelitis, Visna, and cerebral malaria.

[0367] Additional neurologic diseases which can be treated or detected with polynucleotides, polypeptides, agonists, and/or antagonists of the present invention include meningitis such as arachnoiditis, aseptic meningitis such as viral meningitis which includes lymphocytic choriomeningitis, Bacterial meningitis which includes Haemophilus Meningitis, Listeria Meningitis, Meningococcal Meningitis such as Waterhouse-

Friderichsen Syndrome, Pneumococcal Meningitis and meningeal tuberculosis, fungal meningitis such as Cryptococcal Meningitis, subdural effusion, meningoencephalitis such as uve-meningoencephalitic syndrome, myelitis such as transverse myelitis, neurosyphilis such as tabes dorsalis, poliomyelitis which includes bulbar poliomyelitis and postpoliomyelitis syndrome, prion diseases (such as Creutzfeldt-Jakob Syndrome, Bovine Spongiform Encephalopathy, Gerstmann-Straussler Syndrome, Kuru, Scrapie), and cerebral toxoplasmosis.

[0368] Additional neurologic diseases which can be treated or detected with polynucleotides, polypeptides, agonists, and/or antagonists of the present invention include central nervous system neoplasms such as brain neoplasms that include cerebellar neoplasms such as infratentorial neoplasms, cerebral ventricle neoplasms such as choroid plexus neoplasms, hypothalamic neoplasms and supratentorial neoplasms, meningeal neoplasms, spinal cord neoplasms which include epidural neoplasms, demyelinating diseases such as Canavan Diseases, diffuse cerebral scleriosis which includes adrenoleukodystrophy, encephalitis periaxialis, globoid cell leukodystrophy, diffuse cerebral sclerosis such as metachromatic leukodystrophy, allergic encephalomyelitis, necrotizing hemorrhagic encephalomyelitis, progressive multifocal leukoencephalopathy, multiple sclerosis, central pontine myelinolysis, transverse myelitis, neuromyelitis optica, Scrapie, Swayback, Chronic Fatigue Syndrome, Visna, High Pressure Nervous Syndrome, Meningism, spinal cord diseases such as amyotonia congenita, amyotrophic lateral sclerosis, spinal muscular atrophy such as Werdnig-Hoffmann Disease, spinal cord compression, spinal cord neoplasms such as epidural neoplasms, syringomyelia, Tabes Dorsalis, Stiff-Man Syndrome, mental retardation such as Angelman Syndrome, Cri-du-Chat Syndrome, De Lange's Syndrome, Down Syndrome, Gangliosidoses such as gangliosidoses G(M1), Sandhoff Disease, Tay-Sachs Disease, Hartnup Disease, homocystinuria, Laurence-Moon- Biedl Syndrome, Lesch-Nyhan Syndrome, Maple Syrup Urine Disease, mucopolidosis such as fucosidosis, neuronal ceroid-lipofuscinosis, oculocerebrorenal syndrome, phenylketonuria such as maternal phenylketonuria, Prader-Willi Syndrome, Rett Syndrome, Rubinstein-Taybi Syndrome, Tuberous Sclerosis, WAGR Syndrome, nervous system abnormalities such as holoprosencephaly, neural tube defects such as anencephaly which includes hydrangencephaly, Arnold-Chairi Deformity,

encephalocele, meningocele, meningomyelocele, spinal dysraphism such as spina bifida cystica and spina bifida occulta.

[0369] Additional neurologic diseases which can be treated or detected with polynucleotides, polypeptides, agonists, and/or antagonists of the present invention include hereditary motor and sensory neuropathies which include Charcot-Marie Disease, Hereditary optic atrophy, Refsum's Disease, hereditary spastic paraplegia, Werdnig-Hoffmann Disease, Hereditary Sensory and Autonomic Neuropathies such as Congenital Analgesia and Familial Dysautonomia, Neurologic manifestations (such as agnosia that include Gerstmann's Syndrome, Amnesia such as retrograde amnesia, apraxia, neurogenic bladder, cataplexy, communicative disorders such as hearing disorders that includes deafness, partial hearing loss, loudness recruitment and tinnitus, language disorders such as aphasia which include agraphia, anomia, broca aphasia, and Wernicke Aphasia, Dyslexia such as Acquired Dyslexia, language development disorders, speech disorders such as aphasia which includes anomia, broca aphasia and Wernicke Aphasia, articulation disorders, communicative disorders such as speech disorders which include dysarthria, echolalia, mutism and stuttering, voice disorders such as aphonia and hoarseness, decerebrate state, delirium, fasciculation, hallucinations, meningism, movement disorders such as angelman syndrome, ataxia, athetosis, chorea, dystonia, hypokinesia, muscle hypotonia, myoclonus, tic, torticollis and tremor, muscle hypertonia such as muscle rigidity such as stiff-man syndrome, muscle spasticity, paralysis such as facial paralysis which includes Herpes Zoster Oticus, Gastroparesis, Hemiplegia, ophthalmoplegia such as diplopia, Duane's Syndrome, Horner's Syndrome, Chronic progressive external ophthalmoplegia such as Kearns Syndrome, Bulbar Paralysis, Tropical Spastic Paraparesis, Paraplegia such as Brown-Sequard Syndrome, quadriplegia, respiratory paralysis and vocal cord paralysis, paresis, phantom limb, taste disorders such as ageusia and dysgeusia, vision disorders such as amblyopia, blindness, color vision defects, diplopia, hemianopsia, scotoma and subnormal vision, sleep disorders such as hypersomnia which includes Kleine-Levin Syndrome, insomnia, and somnambulism, spasm such as trismus, unconsciousness such as coma, persistent vegetative state and syncope and vertigo, neuromuscular diseases such as amyotonia congenita, amyotrophic lateral sclerosis, Lambert-Eaton Myasthenic Syndrome, motor neuron disease, muscular atrophy such as spinal muscular atrophy, Charcot-Marie Disease and Werdnig-Hoffmann Disease,

Postpoliomyelitis Syndrome, Muscular Dystrophy, Myasthenia Gravis, Myotonia Atrophica, Myotonia Confenita, Nemaline Myopathy, Familial Periodic Paralysis, Multiplex Paramyoclonus, Tropical Spastic Paraparesis and Stiff-Man Syndrome, peripheral nervous system diseases such as acrodynia, amyloid neuropathies, autonomic nervous system diseases such as Adie's Syndrome, Barre-Lieou Syndrome, Familial Dysautonomia, Horner's Syndrome, Reflex Sympathetic Dystrophy and Shy-Drager Syndrome, Cranial Nerve Diseases such as Acoustic Nerve Diseases such as Acoustic Neuroma which includes Neurofibromatosis 2, Facial Nerve Diseases such as Facial Neuralgia, Melkersson-Rosenthal Syndrome, ocular motility disorders which includes amblyopia, nystagmus, oculomotor nerve paralysis, ophthalmoplegia such as Duane's Syndrome, Horner's Syndrome, Chronic Progressive External Ophthalmoplegia which includes Kearns Syndrome, Strabismus such as Esotropia and Exotropia, Oculomotor Nerve Paralysis, Optic Nerve Diseases such as Optic Atrophy which includes Hereditary Optic Atrophy, Optic Disk Drusen, Optic Neuritis such as Neuromyelitis Optica, Papilledema, Trigeminal Neuralgia, Vocal Cord Paralysis, Demyelinating Diseases such as Neuromyelitis Optica and Swayback, and Diabetic neuropathies such as diabetic foot.

[0370] Additional neurologic diseases which can be treated or detected with polynucleotides, polypeptides, agonists, and/or antagonists of the present invention include nerve compression syndromes such as carpal tunnel syndrome, tarsal tunnel syndrome, thoracic outlet syndrome such as cervical rib syndrome, ulnar nerve compression syndrome, neuralgia such as causalgia, cervico-brachial neuralgia, facial neuralgia and trigeminal neuralgia, neuritis such as experimental allergic neuritis, optic neuritis, polyneuritis, polyradiculoneuritis and radiculities such as polyradiculitis, hereditary motor and sensory neuropathies such as Charcot-Marie Disease, Hereditary Optic Atrophy, Refsum's Disease, Hereditary Spastic Paraplegia and Werdnig-Hoffmann Disease, Hereditary Sensory and Autonomic Neuropathies which include Congenital Analgesia and Familial Dysautonomia, POEMS Syndrome, Sciatica, Gustatory Sweating and Tetany).

Immune Activity

[0371] Stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, may be useful in treating deficiencies or disorders of the immune system, by activating or inhibiting the proliferation, differentiation, or mobilization (chemotaxis) of

immune cells. Immune cells develop through a process called hematopoiesis, producing myeloid (platelets, red blood cells, neutrophils, and macrophages) and lymphoid (B and T lymphocytes) cells from pluripotent stem cells. The etiology of these immune deficiencies or disorders may be genetic, somatic, such as cancer or some autoimmune disorders, acquired (e.g., by chemotherapy or toxins), or infectious. Moreover, stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, can be used as a marker or detector of a particular immune system disease or disorder.

[0372] Stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, may be useful in treating or detecting deficiencies or disorders of hematopoietic cells. Stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, could be used to increase differentiation and proliferation of hematopoietic cells, including the pluripotent stem cells, in an effort to treat those disorders associated with a decrease in certain (or many) types hematopoietic cells. Examples of immunologic deficiency syndromes include, but are not limited to: blood protein disorders (e.g. agammaglobulinemia, dysgammaglobulinemia), ataxia telangiectasia, common variable immunodeficiency, DiGeorge Syndrome, HIV infection, HTLV-BLV infection, leukocyte adhesion deficiency syndrome, lymphopenia, phagocyte bactericidal dysfunction, severe combined immunodeficiency (SCIDs), Wiskott-Aldrich Disorder, anemia, thrombocytopenia, or hemoglobinuria.

[0373] Moreover, stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, can also be used to modulate hemostatic (the stopping of bleeding) or thrombolytic activity (clot formation). For example, by increasing hemostatic or thrombolytic activity, stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, could be used to treat blood coagulation disorders (e.g., afibrinogenemia, factor deficiencies), blood platelet disorders (e.g. thrombocytopenia), or wounds resulting from trauma, surgery, or other causes. Alternatively, stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, that can decrease hemostatic or thrombolytic activity could be used to inhibit or dissolve clotting, important in the treatment of heart attacks (infarction), strokes, or scarring.

[0374] Stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, may also be useful in treating or detecting autoimmune disorders. Many autoimmune disorders result from inappropriate recognition of self as foreign material by

immune cells. This inappropriate recognition results in an immune response leading to the destruction of the host tissue. Therefore, the administration of stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, that can inhibit an immune response, particularly the proliferation, differentiation, or chemotaxis of T-cells, may be an effective therapy in preventing autoimmune disorders.

[0375] Examples of autoimmune disorders that can be treated or detected include, but are not limited to: Addison's Disease, hemolytic anemia, antiphospholipid syndrome, rheumatoid arthritis, dermatitis, allergic encephalomyelitis, glomerulonephritis, Goodpasture's Syndrome, Graves' Disease, Multiple Sclerosis, Myasthenia Gravis, Neuritis, Ophthalmia, Bullous Pemphigoid, Pemphigus, Polyendocrinopathies, Purpura, Reiter's Disease, Stiff-Man Syndrome, Autoimmune Thyroiditis, Systemic Lupus Erythematosus, Autoimmune Pulmonary Inflammation, Guillain-Barre Syndrome, insulin dependent diabetes mellitus, and autoimmune inflammatory eye disease.

[0376] Similarly, allergic reactions and conditions, such as asthma (particularly allergic asthma) or other respiratory problems, may also be treated by stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin. Moreover, these molecules can be used to treat anaphylaxis, hypersensitivity to an antigenic molecule, or blood group incompatibility.

[0377] Stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, may also be used to treat and/or prevent organ rejection or graft-versus-host disease (GVHD). Organ rejection occurs by host immune cell destruction of the transplanted tissue through an immune response. Similarly, an immune response is also involved in GVHD, but, in this case, the foreign transplanted immune cells destroy the host tissues. The administration of stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, that inhibits an immune response, particularly the proliferation, differentiation, or chemotaxis of T-cells, may be an effective therapy in preventing organ rejection or GVHD.

[0378] Similarly, stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, may also be used to modulate inflammation. For example, stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, may inhibit the proliferation and differentiation of cells involved in an inflammatory response. These molecules can be used to treat inflammatory conditions, both chronic and

acute conditions, including inflammation associated with infection (e.g., septic shock, sepsis, or systemic inflammatory response syndrome (SIRS)), ischemia-reperfusion injury, endotoxin lethality, arthritis, complement-mediated hyperacute rejection, nephritis, cytokine or chemokine induced lung injury, inflammatory bowel disease, Crohn's disease, or resulting from over production of cytokines (e.g., TNF or IL-1.)

Hyperproliferative Disorders

[0379] Stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, can be used to treat or detect hyperproliferative disorders, including neoplasms. Stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, may inhibit the proliferation of the disorder through direct or indirect interactions. Alternatively, stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, may proliferate other cells which can inhibit the hyperproliferative disorder.

[0380] For example, by increasing an immune response, particularly increasing antigenic qualities of the hyperproliferative disorder or by proliferating, differentiating, or mobilizing T-cells, hyperproliferative disorders can be treated. This immune response may be increased by either enhancing an existing immune response, or by initiating a new immune response. Alternatively, decreasing an immune response may also be a method of treating hyperproliferative disorders, such as a chemotherapeutic agent.

[0381] Examples of hyperproliferative disorders that can be treated or detected by stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, include, but are not limited to neoplasms located in the: abdomen, bone, breast, digestive system, liver, pancreas, peritoneum, endocrine glands (adrenal, parathyroid, pituitary, testicles, ovary, thymus, thyroid), eye, head and neck, nervous (central and peripheral), lymphatic system, pelvic, skin, soft tissue, spleen, thoracic, and urogenital.

[0382] Similarly, other hyperproliferative disorders can also be treated or detected by stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin. Examples of such hyperproliferative disorders include, but are not limited to: hypergammaglobulinemia, lymphoproliferative disorders, paraproteinemias, purpura, sarcoidosis, Sezary Syndrome, Waldenström's Macroglobulinemia, Gaucher's Disease,

histiocytosis, and any other hyperproliferative disease, besides neoplasia, located in an organ system listed above.

Cardiovascular Disorders

[0383] Stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, encoding stanniocalcin may be used to treat cardiovascular disorders, including peripheral artery disease, such as limb ischemia.

[0384] Cardiovascular disorders include cardiovascular abnormalities, such as arterio-arterial fistula, arteriovenous fistula, cerebral arteriovenous malformations, congenital heart defects, pulmonary atresia, and Scimitar Syndrome. Congenital heart defects include aortic coarctation, cor triatriatum, coronary vessel anomalies, crisscross heart, dextrocardia, patent ductus arteriosus, Ebstein's anomaly, Eisenmenger complex, hypoplastic left heart syndrome, levocardia, tetralogy of fallot, transposition of great vessels, double outlet right ventricle, tricuspid atresia, persistent truncus arteriosus, and heart septal defects, such as aortopulmonary septal defect, endocardial cushion defects, Lutembacher's Syndrome, trilogly of Fallot, ventricular heart septal defects.

[0385] Cardiovascular disorders also include heart disease, such as arrhythmias, carcinoid heart disease, high cardiac output, low cardiac output, cardiac tamponade, endocarditis (including bacterial), heart aneurysm, cardiac arrest, congestive heart failure, congestive cardiomyopathy, paroxysmal dyspnea, cardiac edema, heart hypertrophy, congestive cardiomyopathy, left ventricular hypertrophy, right ventricular hypertrophy, post-infarction heart rupture, ventricular septal rupture, heart valve diseases, myocardial diseases, myocardial ischemia, pericardial effusion, pericarditis (including constrictive and tuberculous), pneumopericardium, postpericardiotomy syndrome, pulmonary heart disease, rheumatic heart disease, ventricular dysfunction, hyperemia, cardiovascular pregnancy complications, Scimitar Syndrome, cardiovascular syphilis, and cardiovascular tuberculosis.

[0386] Arrhythmias include sinus arrhythmia, atrial fibrillation, atrial flutter, bradycardia, extrasystole, Adams-Stokes Syndrome, bundle-branch block, sinoatrial block, long QT syndrome, parasystole, Lown-Ganong-Levine Syndrome, Mahaim-type pre-excitation syndrome, Wolff-Parkinson-White syndrome, sick sinus syndrome, tachycardias, and ventricular fibrillation. Tachycardias include paroxysmal tachycardia,

supraventricular tachycardia, accelerated idioventricular rhythm, atrioventricular nodal reentry tachycardia, ectopic atrial tachycardia, ectopic junctional tachycardia, sinoatrial nodal reentry tachycardia, sinus tachycardia, Torsades de Pointes, and ventricular tachycardia.

[0387] Heart valve disease include aortic valve insufficiency, aortic valve stenosis, heart murmurs, aortic valve prolapse, mitral valve prolapse, tricuspid valve prolapse, mitral valve insufficiency, mitral valve stenosis, pulmonary atresia, pulmonary valve insufficiency, pulmonary valve stenosis, tricuspid atresia, tricuspid valve insufficiency, and tricuspid valve stenosis.

[0388] Myocardial diseases include alcoholic cardiomyopathy, congestive cardiomyopathy, hypertrophic cardiomyopathy, aortic subvalvular stenosis, pulmonary subvalvular stenosis, restrictive cardiomyopathy, Chagas cardiomyopathy, endocardial fibroelastosis, endomyocardial fibrosis, Kearns Syndrome, myocardial reperfusion injury, and myocarditis.

[0389] Myocardial ischemias include coronary disease, such as angina pectoris, coronary aneurysm, coronary arteriosclerosis, coronary thrombosis, coronary vasospasm, myocardial infarction and myocardial stunning.

[0390] Cardiovascular diseases also include vascular diseases such as aneurysms, angiodysplasia, angiomas, bacillary angiomas, Hippel-Lindau Disease, Klippel-Trenaunay-Weber Syndrome, Sturge-Weber Syndrome, angioneurotic edema, aortic diseases, Takayasu's Arteritis, aortitis, Leriche's Syndrome, arterial occlusive diseases, arteritis, enarteritis, polyarteritis nodosa, cerebrovascular disorders, diabetic angiopathies, diabetic retinopathy, embolisms, thrombosis, erythromelalgia, hemorrhoids, hepatic veno-occlusive disease, hypertension, hypotension, ischemia, peripheral vascular diseases, phlebitis, pulmonary veno-occlusive disease, Raynaud's disease, CREST syndrome, retinal vein occlusion, Scimitar syndrome, superior vena cava syndrome, telangiectasia, ataxia telangiectasia, hereditary hemorrhagic telangiectasia, varicocele, varicose veins, varicose ulcer, vasculitis, and venous insufficiency.

[0391] Aneurysms include dissecting aneurysms, false aneurysms, infected aneurysms, ruptured aneurysms, aortic aneurysms, cerebral aneurysms, coronary aneurysms, heart aneurysms, and iliac aneurysms.

[0392] Arterial occlusive diseases include arteriosclerosis, intermittent claudication, carotid stenosis, fibromuscular dysplasias, mesenteric vascular occlusion, Moyamoya disease, renal artery obstruction, retinal artery occlusion, and thromboangiitis obliterans.

[0393] Cerebrovascular disorders include carotid artery diseases, cerebral amyloid angiopathy, cerebral aneurysm, cerebral anoxia, cerebral arteriosclerosis, cerebral arteriovenous malformation, cerebral artery diseases, cerebral embolism and thrombosis, carotid artery thrombosis, sinus thrombosis, Wallenberg's syndrome, cerebral hemorrhage, epidural hematoma, subdural hematoma, subarachnoid hemorrhage, cerebral infarction, cerebral ischemia (including transient), subclavian steal syndrome, periventricular leukomalacia, vascular headache, cluster headache, migraine, and vertebrobasilar insufficiency.

[0394] Embolisms include air embolisms, amniotic fluid embolisms, cholesterol embolisms, blue toe syndrome, fat embolisms, pulmonary embolisms, and thromboembolisms. Thrombosis include coronary thrombosis, hepatic vein thrombosis, retinal vein occlusion, carotid artery thrombosis, sinus thrombosis, Wallenberg's syndrome, and thrombophlebitis.

[0395] Ischemia includes cerebral ischemia, ischemic colitis, compartment syndromes, anterior compartment syndrome, myocardial ischemia, reperfusion injuries, and peripheral limb ischemia. Vasculitis includes aortitis, arteritis, Behcet's Syndrome, Churg-Strauss Syndrome, mucocutaneous lymph node syndrome, thromboangiitis obliterans, hypersensitivity vasculitis, Schoenlein-Henoch purpura, allergic cutaneous vasculitis, and Wegener's granulomatosis.

[0396] Stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, are especially effective for the treatment of critical limb ischemia and coronary disease. As shown in the Examples, administration of stanniocalcin polynucleotides and polypeptides to an experimentally induced ischemia rabbit hindlimb may restore blood pressure ratio, blood flow, angiographic score, and capillary density.

[0397] Stanniocalcin polypeptides may be administered using any method known in the art, including, but not limited to, direct needle injection at the delivery site, intravenous injection, topical administration, catheter infusion, biolistic injectors, particle accelerators, gelfoam sponge depots, other commercially available depot materials, osmotic pumps, oral or suppository solid pharmaceutical formulations, decanting or topical applications

during surgery, aerosol delivery. Such methods are known in the art. stanniocalcin polypeptides may be administered as part of a pharmaceutical composition, described in more detail below. Methods of delivering stanniocalcin polynucleotides are described in more detail herein.

Anti-Angiogenesis Activity

[0398] The naturally occurring balance between endogenous stimulators and inhibitors of angiogenesis is one in which inhibitory influences predominate. Rastinejad *et al.*, *Cell* 56:345-355 (1989). In those rare instances in which neovascularization occurs under normal physiological conditions, such as wound healing, organ regeneration, embryonic development, and female reproductive processes, angiogenesis is stringently regulated and spatially and temporally delimited. Under conditions of pathological angiogenesis such as that characterizing solid tumor growth, these regulatory controls fail. Unregulated angiogenesis becomes pathologic and sustains progression of many neoplastic and non-neoplastic diseases. A number of serious diseases are dominated by abnormal neovascularization including solid tumor growth and metastases, arthritis, some types of eye disorders, and psoriasis. See, e.g., reviews by Moses *et al.*, *Biotech.* 9:630-634 (1991); Folkman *et al.*, *N. Engl. J. Med.*, 333:1757-1763 (1995); Auerbach *et al.*, *J. Microvasc. Res.* 29:401-411 (1985); Folkman, *Advances in Cancer Research*, eds. Klein and Weinhouse, Academic Press, New York, pp. 175-203 (1985); Patz, *Am. J. Ophthalmol.* 94:715-743 (1982); and Folkman *et al.*, *Science* 221:719-725 (1983). In a number of pathological conditions, the process of angiogenesis contributes to the disease state. For example, significant data have accumulated which suggest that the growth of solid tumors is dependent on angiogenesis. Folkman and Klagsbrun, *Science* 235:442-447 (1987).

[0399] The present invention provides for treatment of diseases or disorders associated with neovascularization by administration of the stanniocalcin polynucleotides and/or polypeptides of the invention, as well as agonists or antagonists of stanniocalcin. Malignant and metastatic conditions which can be treated with the polynucleotides and polypeptides, or agonists or antagonists of the invention include, but are not limited to, malignancies, solid tumors, and cancers described herein and otherwise known in the art (for a review of such disorders, see Fishman *et al.*, *Medicine*, 2d Ed., J. B. Lippincott Co., Philadelphia (1985)):

[0400] Ocular disorders associated with neovascularization which can be treated with the stanniocalcin polynucleotides and polypeptides of the present invention (including stanniocalcin agonists and/or antagonists) include, but are not limited to: neovascular glaucoma, diabetic retinopathy, retinoblastoma, retrolental fibroplasia, uveitis, retinopathy of prematurity macular degeneration, corneal graft neovascularization, as well as other eye inflammatory diseases, ocular tumors and diseases associated with choroidal or iris neovascularization. See, e.g., reviews by Waltman *et al.*, *Am. J. Ophthalm.* 85:704-710 (1978) and Gartner *et al.*, *Surv. Ophthalm.* 22:291-312 (1978).

[0401] Additionally, disorders which can be treated with the stanniocalcin polynucleotides and polypeptides of the present invention (including stanniocalcin agonist and/or antagonists) include, but are not limited to, hemangioma, arthritis, psoriasis, angiofibroma, atherosclerotic plaques, delayed wound healing, granulations, hemophilic joints, hypertrophic scars, nonunion fractures, Osler-Weber syndrome, pyogenic granuloma, scleroderma, trachoma, and vascular adhesions.

[0402] Moreover, disorders and/or states, which can be treated with be treated with the stanniocalcin polynucleotides and polypeptides of the present invention (including stanniocalcin agonist and/or antagonists) include, but are not limited to, solid tumors, blood born tumors such as leukemias, tumor metastasis, Kaposi's sarcoma, benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas, rheumatoid arthritis, psoriasis, ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis, retinoblastoma, and uvietis, delayed wound healing, endometriosis, vascluogenesis, granulations, hypertrophic scars (keloids), nonunion fractures, scleroderma, trachoma, vascular adhesions, myocardial angiogenesis, coronary collaterals, cerebral collaterals, arteriovenous malformations, ischemic limb angiogenesis, Osler-Webber Syndrome, plaque neovascularization, telangiectasia, hemophiliac joints, angiofibroma fibromuscular dysplasia, wound granulation, Crohn's disease, atherosclerosis, birth control agent by preventing vascularization required for embryo implantation controlling menstruation, diseases that have angiogenesis as a pathologic consequence such as cat scratch disease (*Rochele minalia quintosa*), ulcers (*Helicobacter pylori*), Bartonellosis and bacillary angiomatosis.

Diseases at the Cellular Level

[0403] Diseases associated with increased cell survival or the inhibition of apoptosis that could be treated or detected by stanniocalcin polynucleotides or polypeptides, as well as antagonists or agonists of stanniocalcin, include cancers (such as follicular lymphomas, carcinomas with p53 mutations, and hormone-dependent tumors, including, but not limited to colon cancer, cardiac tumors, pancreatic cancer, melanoma, retinoblastoma, glioblastoma, lung cancer, intestinal cancer, testicular cancer, stomach cancer, neuroblastoma, myxoma, myoma, lymphoma, endothelioma, osteoblastoma, osteoclastoma, osteosarcoma, chondrosarcoma, adenoma, breast cancer, prostate cancer, Kaposi's sarcoma and ovarian cancer); autoimmune disorders (such as, multiple sclerosis, Sjogren's syndrome, Hashimoto's thyroiditis, biliary cirrhosis, Behcet's disease, Crohn's disease, polymyositis, systemic lupus erythematosus and immune-related glomerulonephritis and rheumatoid arthritis) and viral infections (such as herpes viruses, pox viruses and adenoviruses), inflammation, graft v. host disease, acute graft rejection, and chronic graft rejection. In preferred embodiments, stanniocalcin polynucleotides, polypeptides, and/or antagonists of the invention are used to inhibit growth, progression, and/or metastasis of cancers, in particular those listed above.

[0404] Additional diseases or conditions associated with increased cell survival that could be treated or detected by stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, include, but are not limited to, progression, and/or metastases of malignancies and related disorders such as leukemia (including acute leukemias (e.g., acute lymphocytic leukemia, acute myelocytic leukemia (including myeloblastic, promyelocytic, myelomonocytic, monocytic, and erythroleukemia)) and chronic leukemias (e.g., chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia)), polycythemia vera, lymphomas (e.g., Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, and solid tumors including, but not limited to, sarcomas and carcinomas such as fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma,

adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm's tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, and retinoblastoma.

[0405] Diseases associated with increased apoptosis that could be treated or detected by stanniocalcin polynucleotides or polypeptides, as well as agonists or antagonists of stanniocalcin, include AIDS; neurodegenerative disorders (such as Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis, Retinitis pigmentosa, Cerebellar degeneration and brain tumor or prior associated disease); autoimmune disorders (such as, multiple sclerosis, Sjogren's syndrome, Hashimoto's thyroiditis, biliary cirrhosis, Behcet's disease, Crohn's disease, polymyositis, systemic lupus erythematosus and immune-related glomerulonephritis and rheumatoid arthritis) myelodysplastic syndromes (such as aplastic anemia), graft v. host disease, ischemic injury (such as that caused by myocardial infarction, stroke and reperfusion injury), liver injury (e.g., hepatitis related liver injury, ischemia/reperfusion injury, cholestasis (bile duct injury) and liver cancer); toxin-induced liver disease (such as that caused by alcohol), septic shock, cachexia and anorexia.

Wound Healing and Epithelial Cell Proliferation

[0406] In accordance with yet a further aspect of the present invention, there is provided a process for utilizing stanniocalcin polynucleotides or polypeptides, as well as agonists or antagonists of stanniocalcin, for therapeutic purposes, for example, to stimulate epithelial cell proliferation and basal keratinocytes for the purpose of wound healing, and to stimulate hair follicle production and healing of dermal wounds. stanniocalcin polynucleotides or polypeptides, as well as agonists or antagonists of stanniocalcin, may be clinically useful in stimulating wound healing including surgical wounds, excisional wounds, deep wounds involving damage of the dermis and epidermis, eye tissue wounds, dental tissue wounds, oral cavity wounds, diabetic ulcers, dermal ulcers, cubitus ulcers, arterial ulcers, venous stasis ulcers, burns resulting from heat exposure or chemicals, and

other abnormal wound healing conditions such as uremia, malnutrition, vitamin deficiencies and complications associated with systemic treatment with steroids, radiation therapy and antineoplastic drugs and antimetabolites. stanniocalcin polynucleotides or polypeptides, as well as agonists or antagonists of stanniocalcin, could be used to promote dermal reestablishment subsequent to dermal loss

[0407] Stanniocalcin polynucleotides or polypeptides, as well as agonists or antagonists of stanniocalcin, could be used to increase the adherence of skin grafts to a wound bed and to stimulate re-epithelialization from the wound bed. The following are types of grafts that stanniocalcin polynucleotides or polypeptides, agonists or antagonists of stanniocalcin, could be used to increase adherence to a wound bed: autografts, artificial skin, allografts, autodermic graft, autoepidermic grafts, avacular grafts, Blair-Brown grafts, bone graft, brephoplastic grafts, cutis graft, delayed graft, dermic graft, epidermic graft, fascia graft, full thickness graft, heterologous graft, xenograft, homologous graft, hyperplastic graft, lamellar graft, mesh graft, mucosal graft, Ollier-Thiersch graft, omentopial graft, patch graft, pedicle graft, penetrating graft, split skin graft, thick split graft. Stanniocalcin polynucleotides or polypeptides, as well as agonists or antagonists of stanniocalcin, can be used to promote skin strength and to improve the appearance of aged skin.

[0408] It is believed that stanniocalcin polynucleotides or polypeptides, as well as agonists or antagonists of stanniocalcin, will also produce changes in hepatocyte proliferation, and epithelial cell proliferation in the lung, breast, pancreas, stomach, small intestine, and large intestine. Stanniocalcin polynucleotides or polypeptides, as well as agonists or antagonists of stanniocalcin, could promote proliferation of epithelial cells such as sebocytes, hair follicles, hepatocytes, type II pneumocytes, mucin-producing goblet cells, and other epithelial cells and their progenitors contained within the skin, lung, liver, and gastrointestinal tract. Stanniocalcin polynucleotides or polypeptides, agonists or antagonists of stanniocalcin, may promote proliferation of endothelial cells, keratinocytes, and basal keratinocytes.

[0409] Stanniocalcin polynucleotides or polypeptides, as well as agonists or antagonists of stanniocalcin, could also be used to reduce the side effects of gut toxicity that result from radiation, chemotherapy treatments or viral infections. Stanniocalcin polynucleotides or polypeptides, as well as agonists or antagonists of stanniocalcin, may have a cytoprotective effect on the small intestine mucosa. Stanniocalcin polynucleotides or

polypeptides, as well as agonists or antagonists of stanniocalcin, may also stimulate healing of mucositis (mouth ulcers) that result from chemotherapy and viral infections.

[0410] Stanniocalcin polynucleotides or polypeptides, as well as agonists or antagonists of stanniocalcin, could further be used in full regeneration of skin in full and partial thickness skin defects, including burns, (i.e., repopulation of hair follicles, sweat glands, and sebaceous glands), treatment of other skin defects such as psoriasis. Stanniocalcin polynucleotides or polypeptides, as well as agonists or antagonists of stanniocalcin, could be used to treat epidermolysis bullosa, a defect in adherence of the epidermis to the underlying dermis which results in frequent, open and painful blisters by accelerating reepithelialization of these lesions. Stanniocalcin polynucleotides or polypeptides, as well as agonists or antagonists of stanniocalcin, could also be used to treat gastric and duodenal ulcers and help heal by scar formation of the mucosal lining and regeneration of glandular mucosa and duodenal mucosal lining more rapidly. Inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis, are diseases which result in destruction of the mucosal surface of the small or large intestine, respectively. Thus, stanniocalcin polynucleotides or polypeptides, as well as agonists or antagonists of stanniocalcin, could be used to promote the resurfacing of the mucosal surface to aid more rapid healing and to prevent progression of inflammatory bowel disease. Treatment with stanniocalcin polynucleotides or polypeptides, agonists or antagonists of stanniocalcin, is expected to have a significant effect on the production of mucus throughout the gastrointestinal tract and could be used to protect the intestinal mucosa from injurious substances that are ingested or following surgery. Stanniocalcin polynucleotides or polypeptides, as well as agonists or antagonists of stanniocalcin, could be used to treat diseases associate with the under expression of stanniocalcin.

[0411] Moreover, stanniocalcin polynucleotides or polypeptides, as well as agonists or antagonists of stanniocalcin, could be used to prevent and heal damage to the lungs due to various pathological states. A growth factor such as stanniocalcin polynucleotides or polypeptides, as well as agonists or antagonists of stanniocalcin, which could stimulate proliferation and differentiation and promote the repair of alveoli and bronchiolar epithelium to prevent or treat acute or chronic lung damage. For example, emphysema, which results in the progressive loss of alveoli, and inhalation injuries, i.e., resulting from smoke inhalation and burns, that cause necrosis of the bronchiolar epithelium and alveoli

could be effectively treated using stanniocalcin polynucleotides or polypeptides, agonists or antagonists of stanniocalcin. Also, stanniocalcin polynucleotides or polypeptides, as well as agonists or antagonists of stanniocalcin, could be used to stimulate the proliferation of and differentiation of type II pneumocytes, which may help treat or prevent disease such as hyaline membrane diseases, such as infant respiratory distress syndrome and bronchopulmonary dysplasia, in premature infants.

[0412] Stanniocalcin polynucleotides or polypeptides, as well as agonists or antagonists of stanniocalcin, could stimulate the proliferation and differentiation of hepatocytes and, thus, could be used to alleviate or treat liver diseases and pathologies such as fulminant liver failure caused by cirrhosis, liver damage caused by viral hepatitis and toxic substances (i.e., acetaminophen, carbon tetrachloride and other hepatotoxins known in the art).

[0413] In addition, stanniocalcin polynucleotides or polypeptides, as well as agonists or antagonists of stanniocalcin, could be used to treat or prevent the onset of diabetes mellitus. In patients with newly diagnosed Types I and II diabetes, where some islet cell function remains, stanniocalcin polynucleotides or polypeptides, as well as agonists or antagonists of stanniocalcin, could be used to maintain the islet function so as to alleviate, delay or prevent permanent manifestation of the disease. Also, stanniocalcin polynucleotides or polypeptides, as well as agonists or antagonists of stanniocalcin, could be used as an auxiliary in islet cell transplantation to improve or promote islet cell function.

Infectious Disease

[0414] Stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, can be used to treat or detect infectious agents. For example, by increasing the immune response, particularly increasing the proliferation and differentiation of B and/or T cells, infectious diseases may be treated. The immune response may be increased by either enhancing an existing immune response, or by initiating a new immune response. Alternatively, stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, may also directly inhibit the infectious agent, without necessarily eliciting an immune response.

[0415] Viruses are one example of an infectious agent that can cause disease or symptoms that can be treated or detected by stanniocalcin polynucleotides or polypeptides,

or agonists or antagonists of stanniocalcin. Examples of viruses, include, but are not limited to the following DNA and RNA viruses and viral families: Arbovirus, Adenoviridae, Arenaviridae, Arterivirus, Birnaviridae, Bunyaviridae, Caliciviridae, Circoviridae, Coronaviridae, Dengue, EBV, HIV, Flaviviridae, Hepadnaviridae (Hepatitis), Herpesviridae (such as, Cytomegalovirus, Herpes Simplex, Herpes Zoster), Mononegavirus (e.g., Paramyxoviridae, Morbillivirus, Rhabdoviridae), Orthomyxoviridae (e.g., Influenza A, Influenza B, and parainfluenza), Papiloma virus, Papovaviridae, Parvoviridae, Picornaviridae, Poxviridae (such as Smallpox or Vaccinia), Reoviridae (e.g., Rotavirus), Retroviridae (HTLV-I, HTLV-II, Lentivirus), and Togaviridae (e.g., Rubivirus). Viruses falling within these families can cause a variety of diseases or symptoms, including, but not limited to: arthritis, bronchiollitis, respiratory syncytial virus, encephalitis, eye infections (e.g., conjunctivitis, keratitis), chronic fatigue syndrome, hepatitis (A, B, C, E, Chronic Active, Delta), Japanese B encephalitis, Junin, Chikungunya, Rift Valley fever, yellow fever, meningitis, opportunistic infections (e.g., AIDS), pneumonia, Burkitt's Lymphoma, chickenpox, hemorrhagic fever, Measles, Mumps, Parainfluenza, Rabies, the common cold, Polio, leukemia, Rubella, sexually transmitted diseases, skin diseases (e.g., Kaposi's, warts), and viremia. Stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, can be used to treat or detect any of these symptoms or diseases. In specific embodiments, stanniocalcin polynucleotides, polypeptides, agonists or antagonists are used to treat: meningitis, Dengue, EBV, and/or hepatitis (e.g., hepatitis B). In an additional specific embodiment, stanniocalcin polynucleotides, polypeptides, agonists or antagonists are used to treat patients nonresponsive to one or more other commercially available hepatitis vaccines. In a further specific embodiment, stanniocalcin polynucleotides, polypeptides, agonists or antagonists are used to treat AIDS.

[0416] Similarly, bacterial or fungal agents that can cause disease or symptoms and that can be treated or detected by stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, include, but not limited to, the following Gram-Negative and Gram-positive bacteria, bacterial families, and fungi: Actinomyces (e.g., Norcardia), Acinetobacter, *Cryptococcus neoformans*, Aspergillus, Bacillaceae (e.g., *Bacillus anthraxis*), Bacteroides (e.g., *Bacteroides fragilis*), Blastomycosis, Bordetella, Borrelia (e.g., *Borrelia burgdorferi*), Brucella, Candidia, Campylobacter, Chlamydia, Clostridium

(e.g., *Clostridium botulinum*, *Clostridium difficile*, *Clostridium perfringens*, *Clostridium tetani*), Coccidioides, Corynebacterium (e.g., *Corynebacterium diphtheriae*), Cryptococcus, Dermatocycoses, *E. coli* (e.g., Enterotoxigenic *E. coli* and Enterohemorrhagic *E. coli*), Enterobacter (e.g. *Enterobacter aerogenes*), Enterobacteriaceae (Klebsiella, Salmonella (e.g., *Salmonella typhi*, *Salmonella enteritidis*, *Salmonella typhi*), Serratia, Yersinia, Shigella), Erysipelothrix, Haemophilus (e.g., *Haemophilus influenza* type B), Helicobacter, Legionella (e.g., *Legionella pneumophila*), Leptospira, Listeria (e.g., *Listeria monocytogenes*), Mycoplasma, Mycobacterium (e.g., *Mycobacterium leprae* and *Mycobacterium tuberculosis*), Vibrio (e.g., *Vibrio cholerae*), Neisseriaceae (e.g., *Neisseria gonorrhea*, *Neisseria meningitidis*), Pasteurellaceae, Proteus, Pseudomonas (e.g., *Pseudomonas aeruginosa*), Rickettsiaceae, Spirochetes (e.g., Treponema spp., Leptospira spp., Borrelia spp.), Shigella spp., Staphylococcus (e.g., *Staphylococcus aureus*), Meningioccus, Pneumococcus and Streptococcus (e.g., *Streptococcus pneumoniae* and Groups A, B, and C Streptococci), and Ureaplasmas. These bacterial, parasitic, and fungal families can cause diseases or symptoms, including, but not limited to: antibiotic-resistant infections, bacteremia, endocarditis, septicemia, eye infections (e.g., conjunctivitis), uveitis, tuberculosis, gingivitis, bacterial diarrhea, opportunistic infections (e.g., AIDS related infections), paronychia, prosthesis-related infections, dental caries, Reiter's Disease, respiratory tract infections, such as Whooping Cough or Empyema, sepsis, Lyme Disease, Cat-Scratch Disease, dysentery, paratyphoid fever, food poisoning, Legionella disease, chronic and acute inflammation, erythema, yeast infections, typhoid, pneumonia, gonorrhea, meningitis (e.g., meningitis types A and B), chlamydia, syphilis, diphtheria, leprosy, brucellosis, peptic ulcers, anthrax, spontaneous abortions, birth defects, pneumonia, lung infections, ear infections, deafness, blindness, lethargy, malaise, vomiting, chronic diarrhea, Crohn's disease, colitis, vaginosis, sterility, pelvic inflammatory diseases, candidiasis, paratuberculosis, tuberculosis, lupus, botulism, gangrene, tetanus, impetigo, Rheumatic Fever, Scarlet Fever, sexually transmitted diseases, skin diseases (e.g., cellulitis, dermatocycoses), toxemia, urinary tract infections, wound infections, noscomial infections. Stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, can be used to treat or detect any of these symptoms or diseases. In specific embodiments, stanniocalcin polynucleotides,

polypeptides, agonists or antagonists are used to treat: tetanus, diphtheria, botulism, and/or meningitis type B.

[0417] Moreover, parasitic agents causing disease or symptoms that can be treated or detected by stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, include, but not limited to, the following families or classes: Amebiasis, Babesiosis, Coccidiosis, Cryptosporidiosis, Dientamoebiasis, Dourine, Ectoparasitic, Giardiasis, Helminthiasis, Leishmaniasis, Schistosoma, Theileriasis, Toxoplasmosis, Trypanosomiasis, and Trichomonas and Sporozoans (e.g., *Plasmodium virax*, *Plasmodium falciparum*, *Plasmodium malariae* and *Plasmodium ovale*). These parasites can cause a variety of diseases or symptoms, including, but not limited to: Scabies, Trombiculiasis, eye infections, intestinal disease (e.g., dysentery, giardiasis), liver disease, lung disease, opportunistic infections (e.g., AIDS related), malaria, pregnancy complications, and toxoplasmosis. Stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, can be used to treat or detect any of these symptoms or diseases. In specific embodiments, stanniocalcin polynucleotides, polypeptides, agonists or antagonists are used to treat, prevent, and/or diagnose malaria.

[0418] Preferably, treatment using stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, could either be by administering an effective amount of stanniocalcin polypeptide to the patient, or by removing cells from the patient, supplying the cells with stanniocalcin polynucleotide, and returning the engineered cells to the patient (ex vivo therapy). Moreover, the stanniocalcin polypeptide or polynucleotide can be used as an antigen in a vaccine to raise an immune response against infectious disease.

Regeneration

[0419] Stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, can be used to differentiate, proliferate, and attract cells, leading to the regeneration of tissues. (See, Science 276:59-87 (1997).) The regeneration of tissues could be used to repair, replace, or protect tissue damaged by congenital defects, trauma (wounds, burns, incisions, or ulcers), age, disease (e.g. osteoporosis, osteoarthritis, periodontal disease, liver failure), surgery, including cosmetic plastic surgery, fibrosis, reperfusion injury, or systemic cytokine damage.

[0420] Tissues that could be regenerated using the present invention include organs (e.g., pancreas, liver, intestine, kidney, skin, endothelium), muscle (smooth, skeletal or cardiac), vasculature (including vascular and lymphatics), nervous, hematopoietic, and skeletal (bone, cartilage, tendon, and ligament) tissue. Preferably, regeneration occurs without or decreased scarring. Regeneration also may include angiogenesis.

[0421] Moreover, stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, may increase regeneration of tissues difficult to heal. For example, increased tendon/ligament regeneration would quicken recovery time after damage. Stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, of the present invention could also be used prophylactically in an effort to avoid damage. Specific diseases that could be treated include of tendinitis, carpal tunnel syndrome, and other tendon or ligament defects. A further example of tissue regeneration of non-healing wounds includes pressure ulcers, ulcers associated with vascular insufficiency, surgical, and traumatic wounds.

[0422] Similarly, nerve and brain tissue could also be regenerated by using stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, to proliferate and differentiate nerve cells. Diseases that could be treated using this method include central and peripheral nervous system diseases, neuropathies, or mechanical and traumatic disorders (e.g., spinal cord disorders, head trauma, cerebrovascular disease, and stroke). Specifically, diseases associated with peripheral nerve injuries, peripheral neuropathy (e.g., resulting from chemotherapy or other medical therapies), localized neuropathies, and central nervous system diseases (e.g., Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and Shy-Drager syndrome), could all be treated using the stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin.

Chemotaxis

[0423] Stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, may have chemotaxis activity. A chemotaxic molecule attracts or mobilizes cells (e.g., monocytes, fibroblasts, neutrophils, T-cells, mast cells, eosinophils, epithelial and/or endothelial cells) to a particular site in the body, such as inflammation, infection, or

site of hyperproliferation. The mobilized cells can then fight off and/or heal the particular trauma or abnormality.

[0424] Stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, may increase chemotactic activity of particular cells. These chemotactic molecules can then be used to treat inflammation, infection, hyperproliferative disorders, or any immune system disorder by increasing the number of cells targeted to a particular location in the body. For example, chemotactic molecules can be used to treat wounds and other trauma to tissues by attracting immune cells to the injured location. As a chemotactic molecule, stanniocalcin could also attract fibroblasts, which can be used to treat wounds.

[0425] It is also contemplated that stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, may inhibit chemotactic activity. These molecules could also be used to treat disorders. Thus, stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, could be used as an inhibitor of chemotaxis.

Binding Activity

[0426] Stanniocalcin polypeptides may be used to screen for molecules that bind to stanniocalcin or for molecules to which stanniocalcin binds. The binding of stanniocalcin and the molecule may activate (agonist), increase, inhibit (antagonist), or decrease activity of the stanniocalcin or the molecule bound. Examples of such molecules include antibodies, oligonucleotides, proteins (e.g., receptors), or small molecules.

[0427] Preferably, the molecule is closely related to the natural ligand of stanniocalcin, e.g., a fragment of the ligand, or a natural substrate, a ligand, a structural or functional mimetic. (See, Coligan et al., Current Protocols in Immunology 1(2):Chapter 5 (1991).) Similarly, the molecule can be closely related to the natural receptor to which stanniocalcin binds, or at least, a fragment of the receptor capable of being bound by stanniocalcin (e.g., active site). In either case, the molecule can be rationally designed using known techniques.

[0428] Preferably, the screening for these molecules involves producing appropriate cells which express stanniocalcin, either as a secreted protein or on the cell membrane. Preferred cells include cells from mammals, yeast, Drosophila, or E. coli. Cells expressing

stanniocalcin(or cell membrane containing the expressed polypeptide) are then preferably contacted with a test compound potentially containing the molecule to observe binding, stimulation, or inhibition of activity of either stanniocalcin or the molecule.

[0429] The assay may simply test binding of a candidate compound to stanniocalcin, wherein binding is detected by a label, or in an assay involving competition with a labeled competitor. Further, the assay may test whether the candidate compound results in a signal generated by binding to stanniocalcin.

[0430] Alternatively, the assay can be carried out using cell-free preparations, polypeptide/molecule affixed to a solid support, chemical libraries, or natural product mixtures. The assay may also simply comprise the steps of mixing a candidate compound with a solution containing stanniocalcin, measuring stanniocalcin/molecule activity or binding, and comparing the stanniocalcin/molecule activity or binding to a standard.

[0431] Preferably, an ELISA assay can measure stanniocalcin level or activity in a sample (e.g., biological sample) using a monoclonal or polyclonal antibody. The antibody can measure stanniocalcin level or activity by either binding, directly or indirectly, to stanniocalcin or by competing with stanniocalcin for a substrate.

[0432] Additionally, the receptor to which stanniocalcin binds can be identified by numerous methods known to those of skill in the art, for example, ligand panning and FACS sorting (Coligan, et al., Current Protocols in Immun., 1(2), Chapter 5, (1991)). For example, expression cloning is employed wherein polyadenylated RNA is prepared from a cell responsive to the polypeptides, for example, NIH3T3 cells which are known to contain multiple receptors for the FGF family proteins, and SC-3 cells, and a cDNA library created from this RNA is divided into pools and used to transfect COS cells or other cells that are not responsive to the polypeptides. Transfected cells which are grown on glass slides are exposed to the polypeptide of the present invention, after they have been labeled. The polypeptides can be labeled by a variety of means including iodination or inclusion of a recognition site for a site-specific protein kinase.

[0433] Following fixation and incubation, the slides are subjected to auto-radiographic analysis. Positive pools are identified and sub-pools are prepared and re-transfected using an iterative sub-pooling and re-screening process, eventually yielding a single plasmids that encodes the putative receptor.

[0434] As an alternative approach for receptor identification, the labeled polypeptides can be photoaffinity linked with cell membrane or extract preparations that express the receptor molecule. Cross-linked material is resolved by PAGE analysis and exposed to X-ray film. The labeled complex containing the receptors of the polypeptides can be excised, resolved into peptide fragments, and subjected to protein microsequencing. The amino acid sequence obtained from microsequencing would be used to design a set of degenerate oligonucleotide probes to screen a cDNA library to identify the genes encoding the putative receptors.

[0435] Moreover, the techniques of gene-shuffling, motif-shuffling, exon-shuffling, and/or codon-shuffling (collectively referred to as "DNA shuffling") may be employed to modulate the activities of stanniocalcin thereby effectively generating agonists and antagonists of stanniocalcin. *See generally*, U.S. Patent Nos. 5,605,793, 5,811,238, 5,830,721, 5,834,252, and 5,837,458, and Patten, P. A., *et al.*, *Curr. Opinion Biotechnol.* 8:724-33 (1997); Harayama, S. *Trends Biotechnol.* 16(2):76-82 (1998); Hansson, L. O., *et al.*, *J. Mol. Biol.* 287:265-76 (1999); and Lorenzo, M. M. and Blasco, R. *Biotechniques* 24(2):308-13 (1998) (each of these patents and publications are hereby incorporated by reference). In one embodiment, alteration of stanniocalcin polynucleotides and corresponding polypeptides may be achieved by DNA shuffling. DNA shuffling involves the assembly of two or more DNA segments into a desired stanniocalcin molecule by homologous, or site-specific, recombination. In another embodiment, stanniocalcin polynucleotides and corresponding polypeptides may be altered by being subjected to random mutagenesis by error-prone PCR, random nucleotide insertion or other methods prior to recombination. In another embodiment, one or more components, motifs, sections, parts, domains, fragments, etc., of stanniocalcin may be recombined with one or more components, motifs, sections, parts, domains, fragments, etc. of one or more heterologous molecules. In preferred embodiments, the heterologous molecules are stanniocalcin family members. In further preferred embodiments, the heterologous molecule is a growth factor such as, for example, platelet-derived growth factor (PDGF), insulin-like growth factor (IGF-I), transforming growth factor (TGF)-alpha, epidermal growth factor (EGF), fibroblast growth factor (FGF), TGF-beta, bone morphogenetic protein (BMP)-2, BMP-4, BMP-5, BMP-6, BMP-7, activins A and B, decapentaplegic(dpp), 60A, OP-2, dorsalin, growth differentiation factors (GDFs), nodal,

MIS, inhibin-alpha, TGF-beta1, TGF-beta2, TGF-beta3, TGF-beta5, and glial-derived neurotrophic factor (GDNF).

[0436] Other preferred fragments are biologically active stanniocalcin fragments. Biologically active fragments are those exhibiting activity similar, but not necessarily identical, to an activity of the stanniocalcin polypeptide. The biological activity of the fragments may include an improved desired activity, or a decreased undesirable activity.

[0437] Additionally, this invention provides a method of screening compounds to identify those which modulate the action of the polypeptide of the present invention. An example of such an assay comprises combining a mammalian fibroblast cell, a the polypeptide of the present invention, the compound to be screened and $^3\text{[H]}$ thymidine under cell culture conditions where the fibroblast cell would normally proliferate. A control assay may be performed in the absence of the compound to be screened and compared to the amount of fibroblast proliferation in the presence of the compound to determine if the compound stimulates proliferation by determining the uptake of $^3\text{[H]}$ thymidine in each case. The amount of fibroblast cell proliferation is measured by liquid scintillation chromatography which measures the incorporation of $^3\text{[H]}$ thymidine. Both agonist and antagonist compounds may be identified by this procedure.

[0438] In another method, a mammalian cell or membrane preparation expressing a receptor for a polypeptide of the present invention is incubated with a labeled polypeptide of the present invention in the presence of the compound. The ability of the compound to enhance or block this interaction could then be measured. Alternatively, the response of a known second messenger system following interaction of a compound to be screened and the stanniocalcin receptor is measured and the ability of the compound to bind to the receptor and elicit a second messenger response is measured to determine if the compound is a potential agonist or antagonist. Such second messenger systems include but are not limited to, cAMP guanylate cyclase, ion channels or phosphoinositide hydrolysis.

[0439] All of these above assays can be used as diagnostic or prognostic markers. The molecules discovered using these assays can be used to treat disease or to bring about a particular result in a patient (e.g., blood vessel growth) by activating or inhibiting the stanniocalcin molecule. Moreover, the assays can discover agents which may inhibit or enhance the production of stanniocalcin from suitably manipulated cells or tissues. Therefore, the invention includes a method of identifying compounds which bind to

stanniocalcin comprising the steps of: (a) incubating a candidate binding compound with stanniocalcin; and (b) determining if binding has occurred. Moreover, the invention includes a method of identifying agonists/antagonists comprising the steps of: (a) incubating a candidate compound with stanniocalcin, (b) assaying a biological activity, and (c) determining if a biological activity of stanniocalcin has been altered.

[0440] Also, one could identify molecules bind stanniocalcin experimentally by using the beta-pleated sheet regions disclosed in Figure 3 and Table 1. Accordingly, specific embodiments of the invention are directed to polynucleotides encoding polypeptides which comprise, or alternatively consist of, the amino acid sequence of each beta pleated sheet regions disclosed in Figure 3/Table 1. Additional embodiments of the invention are directed to polynucleotides encoding stanniocalcin polypeptides which comprise, or alternatively consist of, any combination or all of the beta pleated sheet regions disclosed in Figure 3/Table 1. Additional preferred embodiments of the invention are directed to polypeptides which comprise, or alternatively consist of, the stanniocalcin amino acid sequence of each of the beta pleated sheet regions disclosed in Figure 3/Table 1. Additional embodiments of the invention are directed to stanniocalcin polypeptides which comprise, or alternatively consist of, any combination or all of the beta pleated sheet regions disclosed in Figure 3/Table 1.

Antisense And Ribozyme (Antagonists)

[0441] In specific embodiments, antagonists according to the present invention are nucleic acids corresponding to the sequences contained in SEQ ID NO:1, or the complementary strand thereof, and/or to nucleotide sequences contained in the deposited plasmid stanniocalcin. In one embodiment, antisense sequence is generated internally by the organism, in another embodiment, the antisense sequence is separately administered (See, for example, O'Connor, *J. Neurochem.*, 56:560 (1991)). Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988). Antisense technology can be used to control gene expression through antisense DNA or RNA, or through triple-helix formation. Antisense techniques are discussed for example, in Okano, *J. Neurochem.*, 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988). Triple helix formation is discussed in, for instance, Lee *et al.*, *Nucleic Acids Research*, 6:3073 (1979); Cooney *et al.*, *Science*,

241:456 (1988); and Dervan *et al.*, *Science*, 251:1300 (1991). The methods are based on binding of a polynucleotide to a complementary DNA or RNA.

[0442] For example, the 5' coding portion of a polynucleotide that encodes the mature polypeptide of the present invention may be used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription thereby preventing transcription and the production of the receptor. The antisense RNA oligonucleotide hybridizes to the mRNA in vivo and blocks translation of the mRNA molecule into receptor polypeptide.

[0443] In one embodiment, the stanniocalcin antisense nucleic acid of the invention is produced intracellularly by transcription from an exogenous sequence. For example, a vector or a portion thereof, is transcribed, producing an antisense nucleic acid (RNA) of the invention. Such a vector would contain a sequence encoding the stanniocalcin antisense nucleic acid. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in vertebrate cells. Expression of the sequence encoding stanniocalcin, or fragments thereof, can be by any promoter known in the art to act in vertebrate, preferably human cells. Such promoters can be inducible or constitutive. Such promoters include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, *Nature* 29:304-310 (1981), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto *et al.*, *Cell*, 22:787-797 (1980), the herpes thymidine promoter (Wagner *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 78:1441-1445 (1981), the regulatory sequences of the metallothionein gene (Brinster *et al.*, *Nature*, 296:39-42 (1982)), etc.

[0444] The antisense nucleic acids of the invention comprise a sequence complementary to at least a portion of an RNA transcript of a stanniocalcin gene. However, absolute complementarity, although preferred, is not required. A sequence "complementary to at least a portion of an RNA," referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double stranded stanniocalcin antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize

will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the larger the hybridizing nucleic acid, the more base mismatches with a stanniocalcin RNA it may contain and still form a stable duplex (or triplex as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

[0445] Oligonucleotides that are complementary to the 5' end of the message, e.g., the 5' untranslated sequence up to and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have been shown to be effective at inhibiting translation of mRNAs as well. See generally, Wagner, R., *Nature*, 372:333-35 (1994). Thus, oligonucleotides complementary to either the 5'- or 3'- non- translated, non-coding regions of stanniocalcin shown in Figures 1A-B could be used in an antisense approach to inhibit translation of endogenous stanniocalcin mRNA. Oligonucleotides complementary to the 5' untranslated region of the mRNA should include the complement of the AUG start codon. Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation but could be used in accordance with the invention. Whether designed to hybridize to the 5'-, 3'- or coding region of stanniocalcin mRNA, antisense nucleic acids should be at least six nucleotides in length, and are preferably oligonucleotides ranging from 6 to about 50 nucleotides in length. In specific aspects the oligonucleotide is at least 10 nucleotides, at least 17 nucleotides, at least 25 nucleotides or at least 50 nucleotides.

[0446] The polynucleotides of the invention can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, etc. The oligonucleotide may include other appended groups such as peptides (e.g., for targeting host cell receptors in vivo), or agents facilitating transport across the cell membrane (See, e.g., Letsinger *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 86:6553-56 (1989); Lemaitre *et al.*, *Proc. Natl. Acad. Sci.*, 84:648-52 (1987); PCT Publication No. WO88/09810) or the blood-brain barrier (see, e.g., PCT Publication No. WO89/10134), hybridization-triggered cleavage agents. (See, e.g., Krol *et al.*, *BioTechniques*, 6:958-976 (1988)) or intercalating agents. (See, e.g., Zon, *Pharm. Res.*, 5:539-549 (1988)). To this end, the oligonucleotide may be conjugated to

another molecule, e.g., a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

[0447] The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including, but not limited to, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

[0448] The antisense oligonucleotide may also comprise at least one modified sugar moiety selected from the group including, but not limited to, arabinose, 2-fluoroarabinose, xylulose, and hexose.

[0449] In yet another embodiment, the antisense oligonucleotide comprises at least one modified phosphate backbone selected from the group including, but not limited to, a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

[0450] In yet another embodiment, the antisense oligonucleotide is an a-anomeric oligonucleotide. An a-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual b-units, the strands run parallel to each other (Gautier et al., *Nucl. Acids Res.* 15:6625-6641 (1987)). The oligonucleotide is a 2'-O-methylribonucleotide (Inoue *et al.*, *Nucl. Acids Res.*, 15:6131-48 (1987)), or a chimeric RNA-DNA analogue (Inoue *et al.*, *FEBS Lett.*, 215:327-30 (1987)).

[0451] Polynucleotides of the invention may be synthesized by standard methods known in the art, e.g. by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate

oligonucleotides may be synthesized by the method of Stein et al. (1988, Nucl. Acids Res. 16:3209), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 85:7448-51 (1988)), etc.

[0452] While antisense nucleotides complementary to the stanniocalcin coding region sequence could be used, those complementary to the transcribed untranslated region are most preferred.

[0453] Potential antagonists according to the invention also include catalytic RNA, or a ribozyme (See, e.g., PCT International Publication WO 90/11364; Sarver *et al.*, *Science*, 247:1222-25 (1990)). While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy stanniocalcin mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Haseloff and Gerlach, *Nature*, 334:585-591 (1988). There are numerous potential hammerhead ribozyme cleavage sites within the nucleotide sequence of stanniocalcin (Figures 1A-B). Preferably, the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the stanniocalcin mRNA; i.e., to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

[0454] As in the antisense approach, the ribozymes of the invention can be composed of modified oligonucleotides (e.g. for improved stability, targeting, etc.) and should be delivered to cells which express stanniocalcin *in vivo*. DNA constructs encoding the ribozyme may be introduced into the cell in the same manner as described above for the introduction of antisense encoding DNA. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive promoter, such as, for example, pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous stanniocalcin messages and inhibit translation. Since ribozymes unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

[0455] Antagonist/agonist compounds may be employed to inhibit the cell growth and proliferation effects of the polypeptides of the present invention on neoplastic cells and tissues, i.e. stimulation of angiogenesis of tumors, and, therefore, retard or prevent abnormal cellular growth and proliferation, for example, in tumor formation or growth.

[0456] The antagonist/agonist may also be employed to prevent hyper-vascular diseases, and prevent the proliferation of epithelial lens cells after extracapsular cataract surgery. Prevention of the mitogenic activity of the polypeptides of the present invention may also be desirable in cases such as restenosis after balloon angioplasty.

[0457] The antagonist/agonist may also be employed to prevent the growth of scar tissue during wound healing.

[0458] The antagonist/agonist may also be employed to treat the diseases described herein.

Other Activities

[0459] The polypeptide of the present invention, as a result of the ability to stimulate vascular endothelial cell growth, may be employed in treatment for stimulating re-vascularization of ischemic tissues due to various disease conditions such as thrombosis, arteriosclerosis, and other cardiovascular conditions. These polypeptide may also be employed to stimulate angiogenesis and limb regeneration, as discussed above.

[0460] The polypeptide may also be employed for treating wounds due to injuries, burns, post-operative tissue repair, and ulcers since they are mitogenic to various cells of different origins, such as fibroblast cells and skeletal muscle cells, and therefore, facilitate the repair or replacement of damaged or diseased tissue.

[0461] The polypeptide of the present invention may also be employed stimulate neuronal growth and to treat and prevent neuronal damage which occurs in certain neuronal disorders or neuro-degenerative conditions such as Alzheimer's disease, Parkinson's disease, and AIDS-related complex. Stanniocalcin may have the ability to stimulate chondrocyte growth, therefore, they may be employed to enhance bone and periodontal regeneration and aid in tissue transplants or bone grafts.

[0462] The polypeptide of the present invention may be also be employed to prevent skin aging due to sunburn by stimulating keratinocyte growth.

[0463] The stanniocalcin polypeptide may also be employed for preventing hair loss, since FGF family members activate hair-forming cells and promotes melanocyte growth. Along the same lines, the polypeptides of the present invention may be employed to stimulate growth and differentiation of hematopoietic cells and bone marrow cells when used in combination with other cytokines.

[0464] The stanniocalcin polypeptide may also be employed to maintain organs before transplantation or for supporting cell culture of primary tissues.

[0465] The polypeptide of the present invention may also be employed for inducing tissue of mesodermal origin to differentiate in early embryos.

[0466] Stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, may also increase or decrease the differentiation or proliferation of embryonic stem cells, besides, as discussed above, hematopoietic lineage.

[0467] Stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, may also be used to modulate mammalian characteristics, such as body height, weight, hair color, eye color, skin, percentage of adipose tissue, pigmentation, size, and shape (e.g., cosmetic surgery). Similarly, stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, may be used to modulate mammalian metabolism affecting catabolism, anabolism, processing, utilization, and storage of energy.

[0468] Stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, may be used to change a mammal's mental state or physical state by influencing biorhythms, cardiac rhythms, depression (including depressive disorders), tendency for violence, tolerance for pain, reproductive capabilities (preferably by Activin or Inhibin-like activity), hormonal or endocrine levels, appetite, libido, memory, stress, or other cognitive qualities.

[0469] Stanniocalcin polynucleotides or polypeptides, or agonists or antagonists of stanniocalcin, may also be used as a food additive or preservative, such as to increase or decrease storage capabilities, fat content, lipid, protein, carbohydrate, vitamins, minerals, cofactors or other nutritional components.

[0470] The above-recited applications have uses in a wide variety of hosts. Such hosts include, but are not limited to, human, murine, rabbit, goat, guinea pig, camel, horse, mouse, rat, hamster, pig, micro-pig, chicken, goat, cow, sheep, dog, cat, non-human

primate, and human. In specific embodiments, the host is a mouse, rabbit, goat, guinea pig, chicken, rat, hamster, pig, sheep, dog or cat. In preferred embodiments, the host is a mammal. In most preferred embodiments, the host is a human.

[0471] Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended as limiting.

[0472] Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended as limiting.

EXAMPLES

Example 1: Stanniocalcin (STC) Protects Neural Cells from the Damaging Effects of Hypoxic Conditions

[0473] The example discloses that treatment of cultivated neural cells with recombinant Stanniocalcin stimulated their uptake of phosphate. Expression of Stanniocalcin by transfection of Stanniocalcin cDNA conferred increased resistance to hypoxic stress and to mobilization of intracellular calcium induced by treatment with thapsigargin. An upregulated and intracellular redistribution of Stanniocalcin expression was seen in human and rat brain neurons in the “penumbra” of infarcted areas. Taken together, these findings indicate that Stanniocalcin plays an important role in maintaining and guarding the integrity of terminally differentiated neuronal cells challenged by ischemia and calcium-mediated cell death.

Methods:

Cell culture and reagents.

[0474] The Paju cell line (Paju/WT) was established in our laboratory from the pleural fluid of a sixteen-year-old girl who had a wide-spread metastatic neural-crest-derived tumor. The cells grow surface adherent in RPMI-1640 medium, supplemented with 10% fetal calf serum, penicillin-G (50 mg/ml), streptomycin sulphate (50 mg/ml), and 1mM glutamine.

[0475] For subculturing, the cells were detached by treatment with 0.5 M EDTA. Human recombinant Stanniocalcin (hSTC) and rabbit antiserum against hSTC were prepared as described. Thapsigargin was purchased from Calbiochem (Calbiochem-Novabiochem Corp. La Jolla, CA, USA).

Cell viability assay and luciferase assay for ATP monitoring.

[0476] Cell viability was assessed by trypan blue exclusion (BDH Chemicals., England). ATP was quantitated by an ATP monitoring kit (BioOrbit, Tampere, Finland) according to the manufacturer's instructions. In brief, cells were lysed in luciferase buffer with 1% Triton X-100 and the ATP-dependent activity was monitored by a luminometer (BioOrbit, Tampere, Finland).

Western blotting.

[0477] Cells were lysed for 10 min. in an ice-cold buffer containing 20 mM Tris/HCl pH8.0, 0.2 mM EDTA, 3% NP40, 2 mM orthovanadate, 50 mM NaF, 10 mM NaPPi, 100 mM NaCl and 10 µg/ml each of aprotinin and leupeptin. The samples were centrifuged at 14000g for 15 min. and the supernatants recovered. Thirty µg protein of each sample was separated by SDS-PAGE under reducing conditions and transferred electrophoretically to nitrocellulose filters. The filters were treated with 3% BSA in 20 mM Tris/HCl pH7.5, 150 mM NaCl, Triton X100 for 2 hrs. Immunoblotting was done with 1:1000 diluted rabbit antibodies to human Stanniocalcin antibody followed by peroxidase-conjugated secondary goat antibodies to anti-rabbit Ig. The blot's were developed by enhanced chemoluminescence (ECL, Amersham, UK).

Immunohistochemistry.

[0478] Tissue was fixed in 4% buffered formaldehyde, routinely processed and embedded in paraffin. Four µm thick sections were mounted on 3-aminopropyltriethoxy-silane (APES)(Sigma, St. Louis, MO, USA) coated slides and dried for 12 hours at 37°C. The deparaffinized and rehydrated sections were processed in a microwave oven and treated with a methanol-perhydrol solution (0.5% hydrogen peroxide in absolute methanol) for 30 minutes at room temperature to block endogenous peroxidase activity. Immuno-histochemical stainings were performed as described. Staining with

Stanniocalcin antibodies preabsorbed with recombinant Stanniocalcin protein and with normal rabbit serum served as controls.

Expression vector constructs and transfection.

[0479] Human Stanniocalcin cDNA containing the full-length open reading frame was cloned into the BamHI site of a pcDNA3 expression vector (Invitrogen). Paju cells were transfected with 5 µg of the vector construct using Lipofectamine Reagent according to the instructions of the manufacturer (GIBCO/BRL). Transfected cells were selected for resistance to G418 (700 µg/ml) for three weeks and single cell cloned (Paju/STC). Control cells were transfected with the empty vector (Paju/C).

Phosphate uptake.

[0480] Phosphate (^{32}Pi) uptake was measured as described at 37°C in Locke's buffer, pH 7.2-7.4, consisting of 5.5 mM KCL, 1.0 mM MgCl_2 , 2.5 mM CaCl_2 , 5.5 mM glucose, 8.5 mM HEPES and 160 mM NaCl. After preincubation in the assay medium for 10 min., the uptake was initiated by addition of 200 ng/ml of recombinant Stanniocalcin together with 125 µM $\text{KH}_2^{32}\text{PO}_4$ (200 µCi/µmol). At indicated time points, ^{32}Pi uptake was terminated by washing with cold stop solution. The cells were lysed in 0.1% SDS in water and the ^{32}Pi activity was measured by liquid scintillation.

Samples of infarcted human brains.

[0481] Specimens were collected at autopsy from the infarcted hemisphere and the corresponding contralateral brain area of patients who had died at different times after the onset of ischemic stroke symptoms. The samples were dissected, processed and histologically analyzed as described in the Helsinki Stroke Study.

Experimental animals and induction of focal brain ischemia.

[0482] Thirty-seven male Wistar rats weighing 310 to 380 g were used.

[0483] Focal cerebral ischemia was induced by the suture occlusion of the medial cerebral artery of anesthetized animals. Reperfusion was established by withdrawing the suture occluder after 90 minutes. The control animals underwent the same procedure, but the suture occluder was inserted only 10 millimeters above the carotid bifurcation and

withdrawn 1 minute later. After postischemia periods of 2 h, 6 h, 24 h, 72 h, and 7 days the experimental animals were anesthetized and subjected to transcardial perfusion with 200 ml of 0.09% saline for 5 min. or until all of the perfusion fluid was clear. The controls underwent transcardiac perfusion 24 hours after the sham occlusion. The brains were immediately removed, 2-millimeter-thick coronal slices were dissected at the level of the optic chiasm, fixated in phosphate-buffered (pH 7) 4% formaldehyde, and embedded in paraffin.

Results:

Elevated extracellular Ca^{2+} induces Stanniocalcin expression in cultivated neural cells.

[0484] Given that elevated environmental calcium is a major trigger of stanniocalcin synthesis in fish (Wagner et al., *Mol Cell Endocrinol.*, 62:31-39 (1989)), we cultivated Paju cells in RPMI-1640 medium containing 5.4 mM of CaCl_2 or MgCl_2 . The expression of Stanniocalcin protein increased strongly after 6 hrs of culture in hypercalcemic medium and reached plateau levels at 12 hrs (Fig. 4). Addition of equimolar concentrations of Mg^{2+} to cultures of Paju cells had no effect on the Stanniocalcin expression (data not shown).

STC stimulates Pi uptake in cultured neural cells.

[0485] Human Stanniocalcin has been reported to stimulate tubular phosphate reabsorption in rat kidney by acting on the renal Na-phosphate co-transporter (Wagner *et al.*, *J. Bone Miner Res.*, 12:165-171 (1997)). Addition of 200 ng/ml recombinant human Stanniocalcin to Paju/WT cells significantly increased their rate of Pi uptake (Fig. 5).

STC confers increased resistance to hypoxia and hypercalcemia

[0486] Treatment with CoCl_2 is commonly used to mimic hypoxic insults *both in vitro*

[0487] and *in vivo*. Paju cells overexpressing Stanniocalcin after transfection with Stanniocalcin cDNA (Paju/STC) and control cells transfected with the empty vector (Paju/C) were cultivated for 12 and 24 hrs in the presence of 300 μM CoCl_2 . Only the Paju/STC cells retained a high viability. The protective role of Stanniocalcin was further substantiated by the fact that in the presence of CoCl_2 , Paju/STC cells maintained an efficient ATP synthesis in comparison to Paju/C cells (Fig. 6).

[0488] Thapsigargin, an inhibitor of Ca^{2+} ATPases in the endoplasmic reticulum mobilizes intracellular calcium stores and leads to increased levels of intracellular free calcium. Paju/STC cells displayed a clearly elevated resistance to treatment with thapsigargin at concentrations toxic to Paju/C cells (Fig. 7).

Transiently upregulated and redistributed neuronal expression of Stanniocalcin in human parietal cortex surrounding brain infarcts.

[0489] We have previously reported that the constitutive expression of Stanniocalcin in mammalian brain is restricted to the neurons and that Immunohistochemistry revealed Stanniocalcin expression mainly in the cell nuclei except for the nucleoli. We also reported that cytoplasm of larger neurons like the pyramidal cells of the parietal cortex, hippocampus and the Purkinje cells of cerebellum and the large neurons in basal nuclei also stained for Stanniocalcin (Zhang *et al.*, *Am J Pathol.*, 153:439-45 (1998)).

[0490] Immunohistochemical stainings of sections from the brain of a patient who died within 15 hours after onset of ischemic stroke revealed a clearly altered distribution of Stanniocalcin in the neurons close to the infarcted area. When compared to neurons in corresponding areas of the contralateral hemisphere, there was an overall increased intensity of staining with a prominent reactivity in the cytoplasm of larger cortical neurons, and in the neuronal processes (Fig. 8 A and B). This increased and redistributed neuronal staining of Stanniocalcin was less apparent in brain sections obtained from a similar location of the ipsilateral hemisphere harboring a three day old ischemic infarct (Fig. 8C). Control stainings with normal rabbit serum or with Stanniocalcin antibodies preabsorbed with recombinant Stanniocalcin protein, gave no neuronal staining (data not shown).

Altered Stanniocalcin expression in experimental ischemic brain insults in rat.

[0491] To further investigate the changes in Stanniocalcin expression in cerebral neurons in response to ischemia we studied brains from rats subjected to experimental transient focal ischemia. In sections from the ischemic core, a slight decrease in Stanniocalcin staining in neurons was seen already after 2 hrs and it decreased in parallel with the maturation of the infarct on the third day. A redistributed and upregulated expression of STC, corresponding to that observed 15 hrs after infarct in human brain, was seen in the 'penumbra' zone surrounding the infarct core. The neurons displayed a strong,

cytoplasmic immunoreactivity for Stanniocalcin which was also translocated to the neuronal processes (Fig. 9). This accentuated and redistributed pattern of Stanniocalcin in neurons of the 'penumbra' area was most prominent at 2 and 6 hrs. It declined gradually and by 7 days returned to the pattern observed in sections of brains from sham operated animals or from the non-infarcted, contralateral hemisphere. The neurons did not stain with normal rabbit serum or antibodies preabsorbed with recombinant Stanniocalcin (data not shown).

Discussion:

[0492] The results indicate that elevated Stanniocalcin expression protects neurons against potentially harmful calcium levels after hypoxia. This notion is supported by our finding that neural cells constitutively overexpressing transfected Stanniocalcin display elevated resistance to treatment with CoCl_2 , which mimics hypoxic stress and leads to influx of calcium. STC-expressing cells also displayed increased resistance to mobilization of intracellular calcium accomplished by treatment with thapsigargin.

[0493] The addition of Stanniocalcin *in vitro* to Paju cells stimulated uptake of Pi. Moreover, it was observed that Paju cells overexpressing Stanniocalcin display a higher steady-state rate of Pi uptake (data not shown). Pi has been shown to buffer intracellular free Ca^{2+} by increasing its sequestration to organelles. These findings are interesting in view of a recent report demonstrating that addition of inorganic phosphate increases neuronal survival *in vitro* during the acute phase after oxidative and excitotoxic insults (Glinn *et al.*, *J Neurochem.*, 70:1850-58 (1998)). Glinn *et al.* reported that Pi influx stimulates ATP synthesis and enhances the energy charge of neurons cultivated from fetal rat brain. Glinn *et al.* also found that neurons pre-exposed to Pi had higher steady state levels of ATP than Pi-starved cells. Elevated stores of high energy phosphate have been found to improve neuronal survival under excitotoxic conditions.

[0494] Despite the lack of measurably increased levels of ATP in stc-transfected or STC-treated cells, Paju/STC cells showed a significantly increased ability to maintain the ATP synthesis in hypoxic environment.

[0495] Thus, the findings disclosed herein demonstrate a previously uncharacterized neurochemical control mechanism where STC, known to regulate calcium and phosphate homeostasis in fish, contributes to the protection of neurons challenged by hypoxia or

ischemia. Similar patterns of Stanniocalcin expression were found in rat and human stroke. The Stanniocalcin compositions of the invention (i.e., Stanniocalcin polynucleotides, polypeptides, and/or agonists or antagonists) therefore offer a novel approach to therapeutic interventions aimed at limiting the damage after brain insults.

[0496] Citation of references herein above shall not be construed as an admission that such references are prior art to the present invention.

Example 2: Isolation of the Stanniocalcin cDNA Clone From the Deposited Sample

[0497] Two approaches can be used to isolate stanniocalcin from the deposited sample. First, the deposited clone is transformed into a suitable host (such as XL-1 Blue (Stratagene)) using techniques known to those of skill in the art, such as those provided by the vector supplier or in related publications or patents. The transformants are plated on 1.5% agar plates (containing the appropriate selection agent, e.g., ampicillin) to a density of about 150 transformants (colonies) per plate. A single colony is then used to generate DNA using nucleic acid isolation techniques well known to those skilled in the art. (e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Edit., (1989), Cold Spring Harbor Laboratory Press.)

[0498] Alternatively, two primers of 17-20 nucleotides derived from both ends of the SEQ ID NO:1 (i.e., within the region of SEQ ID NO:1 bounded by the 5' NT and the 3' NT of the clone) are synthesized and used to amplify the stanniocalcin cDNA using the deposited cDNA plasmid as a template. The polymerase chain reaction is carried out under routine conditions, for instance, in 25 ul of reaction mixture with 0.5 ug of the above cDNA template. A convenient reaction mixture is 1.5-5 mM MgCl₂, 0.01% (w/v) gelatin, 20 uM each of dATP, dCTP, dGTP, dTTP, 25 pmol of each primer and 0.25 Unit of Taq polymerase. Thirty five cycles of PCR (denaturation at 94 degree C for 1 min; annealing at 55 degree C for 1 min; elongation at 72 degree C for 1 min) are performed with a Perkin-Elmer Cetus automated thermal cycler. The amplified product is analyzed by agarose gel electrophoresis and the DNA band with expected molecular weight is excised and purified. The PCR product is verified to be the selected sequence by subcloning and sequencing the DNA product.

[0499] Several methods are available for the identification of the 5' or 3' non-coding portions of the stanniocalcin gene which may not be present in the deposited clone. These methods include but are not limited to, filter probing, clone enrichment using specific probes, and protocols similar or identical to 5' and 3' "RACE" protocols which are well known in the art. For instance, a method similar to 5' RACE is available for generating the missing 5' end of a desired full-length transcript. (Fromont-Racine et al., *Nucleic Acids Res.* 21(7):1683-1684 (1993).)

[0500] Briefly, a specific RNA oligonucleotide is ligated to the 5' ends of a population of RNA presumably containing full-length gene RNA transcripts. A primer set containing a primer specific to the ligated RNA oligonucleotide and a primer specific to a known sequence of the stanniocalcin gene of interest is used to PCR amplify the 5' portion of the stanniocalcin full-length gene. This amplified product may then be sequenced and used to generate the full length gene.

[0501] This above method starts with total RNA isolated from the desired source, although poly-A⁺ RNA can be used. The RNA preparation can then be treated with phosphatase if necessary to eliminate 5' phosphate groups on degraded or damaged RNA which may interfere with the later RNA ligase step. The phosphatase should then be inactivated and the RNA treated with tobacco acid pyrophosphatase in order to remove the cap structure present at the 5' ends of messenger RNAs. This reaction leaves a 5' phosphate group at the 5' end of the cap cleaved RNA which can then be ligated to an RNA oligonucleotide using T4 RNA ligase.

[0502] This modified RNA preparation is used as a template for first strand cDNA synthesis using a gene specific oligonucleotide. The first strand synthesis reaction is used as a template for PCR amplification of the desired 5' end using a primer specific to the ligated RNA oligonucleotide and a primer specific to the known sequence of the gene of interest. The resultant product is then sequenced and analyzed to confirm that the 5' end sequence belongs to the stanniocalcin gene.

Example 3: Bacterial Expression and Purification of Human Stanniocalcin Protein

[0503] The DNA sequence encoding stanniocalcin protein, ATCC # 75652, is initially amplified using PCR oligonucleotide primers corresponding to the 5' and 3' end sequences of the stanniocalcin nucleic acid sequence. Additional nucleotides corresponding to the SphI and BglII restriction enzyme site were added to the 5' and 3' sequences respectively. The 5' oligonucleotide primer has the sequence 5' GACTGCATGCTCCAAACTCAGCAGTG 3' (SEQ ID NO:5), contains a SphI restriction enzyme site and 21 nucleotides of the stanniocalcin protein coding sequence starting from the initiation codon; the 3' sequence 3' GACTAGATCTTGCACCTCTCATGGGATGTGCG 5' (SEQ ID NO:6) contains complementary sequences to a BglII restriction site (AGATCT) and the last 21 nucleotides of the stanniocalcin protein coding sequence. The restriction enzyme sites correspond to the restriction enzyme sites on the bacterial expression vector pQE70 (Qiagen, Inc. Chatsworth, CA). pQE70 encodes antibiotic resistance (Amp^r), a bacterial origin of replication (ori), an IPTG-regulatable promoter operator (P/O), a ribosome binding site (RBS), a 6-His tag and restriction enzyme sites. pQE70 was then digested with the SphI and BglII restriction enzymes. The amplified sequences were ligated into pQE70 and were inserted in frame with the sequence encoding for the histidine tag and the RBS. The ligation mixture was then used to transform E. coli strain M15/rep4 (Qiagen, Inc.) which contains multiple copies of the plasmid pREP4, which expresses the lacI repressor and also confers kanamycin resistance (Kan^r). Transformants are identified by their ability to grow on LB plates and ampicillin/kanamycin resistant colonies were selected. Plasmid DNA was isolated and confirmed by restriction analysis. Clones containing the desired constructs were grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25 ug/ml). The O/N culture is used to inoculate a large culture at a ratio of 1:100 to 1:250. The cells were grown to an optical density 600 (O.D.₆₀₀) of between 0.4 and 0.6. IPTG (Isopropyl-B-D-thiogalacto pyranoside) was then added to a final concentration of 1 mM. IPTG induces by inactivating the lacI repressor, clearing the P/O leading to increased gene expression. Cells were grown an extra 3 to 4

hours. Cells were then harvested by centrifugation (20 mins at 6000Xg). The cell pellet was solubilized in the chaotropic agent 6 Molar Guanidine HCl. After clarification, solubilized stanniocalcin was purified from this solution by chromatography on a Nickel-Chelate column under conditions that allow for tight binding by proteins containing the 6-His tag (Hochuli, E. et al., Genetic Engineering, Principles & Methods, 12:87-98 (1990). Protein renaturation out of GnHCl can be accomplished by several protocols (Jaenicke, R. and Rudolph, R., Protein Structure - A Practical Approach, IRL Press, New York (1990)). Initially, step dialysis is utilized to remove the GnHCl. Alternatively, the purified protein isolated from the Ni-chelate column can be bound to a second column over which a decreasing linear GnHCL gradient is run. The protein is allowed to renature while bound to the column and is subsequently eluted with a buffer containing 250 mM Imidazole, 150 mM NaCl, 25 mM Tris-HCl pH 7.5 and 10% Glycerol. Finally, soluble protein is dialyzed against a storage buffer containing 5 mM Ammonium Bicarbonate.

Example 4: Cloning and expression of Human Stanniocalcin using the baculovirus expression system

[0504] The DNA sequence encoding the full length human Stanniocalcin protein, ATCC # 75652, was amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene, as described in Example 2. The 5' primer has the sequence 5' CAGTGGATCCGCCACCATGCTCCAAACTCAGCAGTG 3' (SEQ ID NO:7) and contains a BamHI restriction enzyme site followed by 6 nucleotides resembling an efficient signal for the initiation of translation in eukaryotic cells (Kozak, M., J. Mol. Biol., 196:947-950 (1987) which is just behind the first 21 nucleotides of the human stanniocalcin gene (the initiation codon for translation "ATG" is underlined). The 3' primer has the sequence 5' CAGTGGTACCGGTTGTGAATAACCTCTCCC 3' (SEQ ID NO:8) and contains the cleavage site for the restriction endonuclease Asp718 and 20 nucleotides complementary to the 3' non-translated sequence of the stanniocalcin gene. The fragment was digested with the endonucleases BamHI and Asp718 and then purified again on a 1% agarose gel. This fragment is designated F2.

[0505] The vector pRG1 (modification of pVL941 vector, discussed below) is used for the expression of the human stanniocalcin protein using the baculovirus expression system

(for review see: Summers, M.D. and Smith, G.E. 1987, A manual of methods for baculovirus vectors and insect cell culture procedures, Texas Agricultural Experimental Station Bulletin No. 1555). This expression vector contains the strong polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) followed by the recognition sites for the restriction endonucleases BamHI and Asp718. The polyadenylation site of the simian virus (SV)40 is used for efficient polyadenylation. For an easy selection of recombinant virus the beta-galactosidase gene from *E.coli* is inserted in the same orientation as the polyhedrin promoter followed by the polyadenylation signal of the polyhedrin gene. The polyhedrin sequences are flanked at both sides by viral sequences for the cell-mediated homologous recombination of co-transfected wild-type viral DNA. Many other baculovirus vectors could be used in place of pRG1 such as pAc373, pVL941 and pAcIM1 (Luckow, V.A. and Summers, M.D., *Virology*, 170:31-39).

[0506] The plasmid was digested with the restriction enzymes BamHI and Asp718 and then dephosphorylated using calf intestinal phosphatase by procedures known in the art. The DNA was then isolated from a 1% agarose gel using the commercially available kit ("GeneClean" BIO 101 Inc., La Jolla, Ca.). This vector DNA is designated V2.

[0507] Fragment F2 and the dephosphorylated plasmid V2 were ligated with T4 DNA ligase. *E.coli* HB101 cells were then transformed and bacteria identified that contained the plasmid (pBac-hSTC) with the human stanniocalcin gene using the enzymes BamHI and Asp718. The sequence of the cloned fragment was confirmed by DNA sequencing.

[0508] 5 μ g of the plasmid pBac-hSTC was co-transfected with 1.0 μ g of a commercially available linearized baculovirus ("BaculoGold baculovirus DNA", Pharmingen, San Diego, CA.) using the lipofection method (Felgner et al. *Proc. Natl. Acad. Sci. USA*, 84:7413-7417 (1987)).

[0509] 1 μ g of BaculoGold virus DNA and 5 μ g of the plasmid pBac-hSTC were mixed in a sterile well of a microtiter plate containing 50 μ l of serum free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards 10 μ l Lipofectin plus 90 μ l Grace's medium were added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture was added drop-wise to the Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 ml Grace's medium without serum. The plate was rocked back and forth to mix the newly added solution. The plate was then incubated for 5 hours at 27°C. After 5 hours the transfection solution was removed from

the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum was added. The plate was put back into an incubator and cultivation continued at 27°C for four days.

[0510] After four days the supernatant was collected and a plaque assay performed similar as described by Summers and Smith (supra). As a modification an agarose gel with “Blue Gal” (Life Technologies Inc., Gaithersburg) was used which allows an easy isolation of blue stained plaques. (A detailed description of a “plaque assay” can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10).

[0511] Four days after the serial dilution, the virus was added to the cells and blue stained plaques were picked with the tip of an Eppendorf pipette. The agar containing the recombinant viruses was then resuspended in an Eppendorf tube containing 200 μ l of Grace's medium. The agar was removed by a brief centrifugation and the supernatant containing the recombinant baculovirus was used to infect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes were harvested and then stored at 4°C.

[0512] Sf9 cells were grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells were infected with the recombinant baculovirus V-hSTC at a multiplicity of infection (MOI) of 2. Six hours later the medium was removed and replaced with SF900 II medium minus methionine and cysteine (Life Technologies Inc., Rockville). 42 hours later 5 μ Ci of 35 S-methionine and 5 μ Ci 35 S cysteine (Amersham) were added. The cells were further incubated for 16 hours before they were harvested by centrifugation and the labeled proteins visualized by SDS-PAGE and autoradiography.

Example 5: Cloning and expression of human Stanniocalcin Protein using Chinese Hamster Ovary Cells Lacking Dihydrofolate Activity

[0513] The vector pN346 is used for the expression of the human stanniocalcin protein. Plasmid pN346 is a derivative of the plasmid pSV2-DHFR [ATCC Accession No. 37146]. Both plasmids contain the mouse dihydrofolate reductase (DHFR) gene under control of the SV40 early promoter. Chinese hamster ovary, or other cells, lacking dihydrofolate activity that are transfected with these plasmids can be selected by growing the cells in a

selective medium (alpha minus MEM, Life Technologies) supplemented with the chemotherapeutic agent methotrexate (MTX). The amplification of the DHFR genes in cells resistant to methotrexate has been well documented (see, e.g., Alt, F.W., Kellems, R.M., Bertino, J.R., and Schimke, R.T., 1978, J. Biol. Chem. 253:1357-1370, Hamlin, J.L. and Ma, C. 1990, Biochem. et Biophys. Acta, 1097:107-143, Page, M.J. and Sydenham, M.A. 1991, Biotechnology Vol. 9:64-68). Cells grown in increasing concentrations of MTX develop resistance to the drug by overproducing the target enzyme, DHFR, as a result of amplification of the DHFR gene. If a second gene is linked to the DHFR gene it is usually co-amplified and over-expressed. It is state of the art to develop cell lines carrying more than 1000 copies of the genes. Subsequently, when the methotrexate is withdrawn, cell lines contain the amplified gene integrated into the chromosome(s).

[0514] Plasmid pN346 contains a strong promoter for the expression of the gene of interest, namely, the long terminal repeat (LTR) of the Rous Sarcoma Virus (Cullen et al., Molecular and Cellular Biology, March 1985, 438-447) plus a fragment isolated from the enhancer of the immediate early gene of human cytomegalovirus (CMV) (Boshart et al., Cell 41:521-530, 1985). Downstream of the promoter are the following single restriction enzyme cleavage sites that allow the integration of the genes; BamHI, PvuII, and NruI. Behind these cloning sites, the plasmid contains translational stop codons in all three reading frames followed by the 3' intron and the polyadenylation site of the rat preproinsulin gene. Other high efficient promoters can also be used for expression, e.g., the human -actin promoter, the SV40 early or late promoters or the long terminal repeats from other retroviruses, e.g., HIV and HTLV. For the polyadenylation of mRNA, other signals, e.g., from the human growth hormone or globin genes, may be used as well.

[0515] Stable cell lines carrying a gene of interest integrated into the chromosome can also be selected upon co-transfection with a selectable marker such as gpt, G418 or hygromycin. It is advantageous to use more than one selectable marker in the beginning, e.g., G418 plus methotrexate.

[0516] The plasmid pN346 was digested with the restriction enzyme BamHI and then dephosphorylated using calf intestinal phosphatase by procedures known in the art. The vector was then isolated from a 1% agarose gel.

[0517] The DNA sequence encoding human stanniocalcin protein, ATCC # 75652, was amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of

the gene. The 5' primer has the sequence 5' CAGTGGATCCGCCACCATGCTCCAAACTCAGCAGTG 3' (SEQ ID NO:9) and contains a BamHI restriction enzyme site followed by 6 nucleotides resembling the efficient signal for translation (Kozak, M., supra) plus the first 21 nucleotides of the gene (the initiation codon for translation "ATG" is underlined.) The 3' primer has the sequence 5' CAGTGGATCCGGTTGTGAATAACCTCTCCC 3' (SEQ ID NO:10) and contains the cleavage site for the restriction endonuclease BamHI and 20 nucleotides complementary to the 3' non-translated sequence of the gene. The amplified fragments were digested with the endonuclease BamHI and then purified on a 1% agarose gel.

[0518] The isolated fragment and the dephosphorylated vector were then ligated with T4 DNA ligase. E.coli HB101 cells were then transformed and bacteria identified that contained the plasmid pN346hSTC inserted in the correct orientation. The sequence of the inserted gene was confirmed by DNA sequencing.

Transfection of CHO-DHFR-cells

[0519] Chinese hamster ovary cells lacking an active DHFR enzyme were used for transfection. 5 μ g of the expression plasmid pN346hSTC were co-transfected with 0.5 μ g of the plasmid pSVneo using the lipofection method (Felgner et al., supra). The plasmid pSV2-neo contains a dominant selectable marker, the gene *neo* from Tn5 encoding an enzyme that confers resistance to a group of antibiotics including G418. The cells were seeded in alpha minus MEM supplemented with 1 mg/ml G418. After 2 days, the cells were trypsinized and seeded in hybridoma cloning plates (Greiner, Germany) and cultivated for 10-14 days. After this period, single clones were trypsinized and then seeded in 6-well petri dishes using different concentrations of methotrexate (25, 50 nM, 100 nM, 200 nM, 400 nM). Clones growing at the highest concentrations of methotrexate were then transferred to new 6-well plates containing even higher concentrations of methotrexate (500 nM, 1 μ M, 2 μ M, 5 μ M). The same procedure was repeated until clones grew at a concentration of 100 μ M.

[0520] The expression of the desired gene product was analyzed by Western blot analysis and SDS-PAGE.

***Example 6: Construction of N-Terminal and/or
C-Terminal Deletion Mutants***

[0521] The following general approach may be used to clone a N-terminal or C-terminal deletion stanniocalcin deletion mutant. Generally, two oligonucleotide primers of about 15-25 nucleotides are derived from the desired 5' and 3' positions of a polynucleotide of SEQ ID NO:1. The 5' and 3' positions of the primers are determined based on the desired stanniocalcin polynucleotide fragment. An initiation and stop codon are added to the 5' and 3' primers respectively, if necessary, to express the stanniocalcin polypeptide fragment encoded by the polynucleotide fragment. Preferred stanniocalcin polynucleotide fragments are those encoding the N-terminal and C-terminal deletion mutants disclosed above in the "Polynucleotide and Polypeptide Fragments" section of the Specification.

[0522] Additional nucleotides containing restriction sites to facilitate cloning of the stanniocalcin polynucleotide fragment in a desired vector may also be added to the 5' and 3' primer sequences. The stanniocalcin polynucleotide fragment is amplified from genomic DNA or from the deposited cDNA clone using the appropriate PCR oligonucleotide primers and conditions discussed herein or known in the art. The stanniocalcin polypeptide fragments encoded by the stanniocalcin polynucleotide fragments of the present invention may be expressed and purified in the same general manner as the full length polypeptides, although routine modifications may be necessary due to the differences in chemical and physical properties between a particular fragment and full length polypeptide.

[0523] As a means of exemplifying but not limiting the present invention, the polynucleotide encoding the stanniocalcin polypeptide fragment F-57 to F-108 is amplified and cloned as follows: A 5' primer is generated comprising a restriction enzyme site followed by an initiation codon in frame with the polynucleotide sequence encoding the N-terminal portion of the polypeptide fragment beginning with F-57. A complementary 3' primer is generated comprising a restriction enzyme site followed by a stop codon in frame with the polynucleotide sequence encoding C-terminal portion of the stanniocalcin polypeptide fragment ending with F-108.

[0524] The amplified polynucleotide fragment and the expression vector are digested with restriction enzymes which recognize the sites in the primers. The digested

polynucleotides are then ligated together. The stanniocalcin polynucleotide fragment is inserted into the restricted expression vector, preferably in a manner which places the stanniocalcin polypeptide fragment coding region downstream from the promoter. The ligation mixture is transformed into competent *E. coli* cells using standard procedures and as described in the Examples herein. Plasmid DNA is isolated from resistant colonies and the identity of the cloned DNA confirmed by restriction analysis, PCR and DNA sequencing.

Example 7: Protein Fusions of Stanniocalcin

[0525] Stanniocalcin polypeptides are preferably fused to other proteins. These fusion proteins can be used for a variety of applications. For example, fusion of stanniocalcin polypeptides to His-tag, HA-tag, protein A, IgG domains, and maltose binding protein facilitates purification. (See Example 5; see also EP A 394,827; Traunecker, et al., *Nature* 331:84-86 (1988).) Similarly, fusion to IgG-1, IgG-3, and albumin increases the half-life time in vivo. Nuclear localization signals fused to stanniocalcin polypeptides can target the protein to a specific subcellular localization, while covalent heterodimer or homodimers can increase or decrease the activity of a fusion protein. Fusion proteins can also create chimeric molecules having more than one function. Finally, fusion proteins can increase solubility and/or stability of the fused protein compared to the non-fused protein. All of the types of fusion proteins described above can be made by modifying the following protocol, which outlines the fusion of a polypeptide to an IgG molecule.

[0526] Briefly, the human Fc portion of the IgG molecule can be PCR amplified, using primers that span the 5' and 3' ends of the sequence described below. These primers also should have convenient restriction enzyme sites that will facilitate cloning into an expression vector, preferably a mammalian expression vector.

[0527] For example, if pC4 (Accession No. 209646) is used, the human Fc portion can be ligated into the BamHI cloning site. Note that the 3' BamHI site should be destroyed. Next, the vector containing the human Fc portion is re-restricted with BamHI, linearizing the vector, and stanniocalcin polynucleotide, isolated by the PCR protocol described in Example 1, is ligated into this BamHI site. Note that the polynucleotide is cloned without a stop codon, otherwise a fusion protein will not be produced.

[0528] If the naturally occurring signal sequence is used to produce the secreted protein, pC4 does not need a second signal peptide. Alternatively, if the naturally occurring signal sequence is not used, the vector can be modified to include a heterologous signal sequence. (See, e.g., WO 96/34891.)

Human IgG Fc region:

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GGGATCCGGAGCCCAAATCTTCTGACAAAACCTCACACATGCCACCGTGCCAGCACCTGAATT
CGAGGGTGCACCGTCAGTCTTCCTCTTCCCCCAAACCCAAGGACACCCTCATGATCTCCCGG
ACTCCTGAGGTACATGCGTGGTGGTGGACGTAAGCCACGAAGACCCTGAGGTCAAGTTCAAC
TGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAAC
AGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGT
ACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAACCCCATCGAGAAAACCATCTCCAAAGCCAA
AGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAA
CCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCAAGCGACATCGCCGTGGAGTGGGAG
AGCAATGGGCAGCCGGAGAACAACACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCC
TTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCAT
GCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGG
TAAATGAGTGCGACGGCCGCGACTCTAGAGGAT (SEQ ID NO:4)
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Example 8: Production of an Antibody

Hybridoma Technology

[0529] The antibodies of the present invention can be prepared by a variety of methods. (See, Current Protocols, Chapter 2.) As one example of such methods, cells expressing stanniocalcin are administered to an animal to induce the production of sera containing polyclonal antibodies. In a preferred method, a preparation of stanniocalcin protein is prepared and purified to render it substantially free of natural contaminants. Such a preparation is then introduced into an animal in order to produce polyclonal antisera of greater specific activity.

[0530] In the most preferred method, the antibodies of the present invention are monoclonal antibodies (or protein binding fragments thereof). Such monoclonal antibodies can be prepared using hybridoma technology. (Köhler et al., Nature 256:495 (1975); Köhler et al., Eur. J. Immunol. 6:511 (1976); Köhler et al., Eur. J. Immunol. 6:292 (1976); Hammerling et al., in: Monoclonal Antibodies and T-Cell Hybridomas, Elsevier,

N.Y., pp. 563-681 (1981).) In general, such procedures involve immunizing an animal (preferably a mouse) with stanniocalcin polypeptide or, more preferably, with a secreted stanniocalcin polypeptide-expressing cell. Such cells may be cultured in any suitable tissue culture medium; however, it is preferable to culture cells in Earle's modified Eagle's medium supplemented with 10% fetal bovine serum (inactivated at about 56 degree C), and supplemented with about 10 g/l of nonessential amino acids, about 1,000 U/ml of penicillin, and about 100 ug/ml of streptomycin.

[0531] The splenocytes of such mice are extracted and fused with a suitable myeloma cell line. Any suitable myeloma cell line may be employed in accordance with the present invention; however, it is preferable to employ the parent myeloma cell line (SP2O), available from the ATCC. After fusion, the resulting hybridoma cells are selectively maintained in HAT medium, and then cloned by limiting dilution as described by Wands et al. (Gastroenterology 80:225-232 (1981).) The hybridoma cells obtained through such a selection are then assayed to identify plasmids which secrete antibodies capable of binding the stanniocalcin polypeptide.

[0532] Alternatively, additional antibodies capable of binding to stanniocalcin polypeptide can be produced in a two-step procedure using anti-idiotypic antibodies. Such a method makes use of the fact that antibodies are themselves antigens, and therefore, it is possible to obtain an antibody which binds to a second antibody. In accordance with this method, protein specific antibodies are used to immunize an animal, preferably a mouse. The splenocytes of such an animal are then used to produce hybridoma cells, and the hybridoma cells are screened to identify plasmids which produce an antibody whose ability to bind to the stanniocalcin protein-specific antibody can be blocked by stanniocalcin. Such antibodies comprise anti-idiotypic antibodies to the stanniocalcin protein-specific antibody and can be used to immunize an animal to induce formation of further stanniocalcin protein-specific antibodies.

[0533] It will be appreciated that Fab and F(ab')₂ and other fragments of the antibodies of the present invention may be used according to the methods disclosed herein. Such fragments are typically produced by proteolytic cleavage, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')₂ fragments). Alternatively, secreted stanniocalcin protein-binding fragments can be produced through the application of recombinant DNA technology or through synthetic chemistry.

[0534] For in vivo use of antibodies in humans, it may be preferable to use “humanized” chimeric monoclonal antibodies. Such antibodies can be produced using genetic constructs derived from hybridoma cells producing the monoclonal antibodies described above. Methods for producing chimeric antibodies are known in the art. (See, for review, Morrison, Science 229:1202 (1985); Oi et al., BioTechniques 4:214 (1986); Cabilly et al., U.S. Patent No. 4,816,567; Taniguchi et al., EP 171496; Morrison et al., EP 173494; Neuberger et al., WO 8601533; Robinson et al., WO 8702671; Boulianne et al., Nature 312:643 (1984); Neuberger et al., Nature 314:268 (1985).)

Isolation of antibody fragments directed against stanniocalcin from a library of scFvs.

[0535] Naturally occurring V-genes isolated from human PBLs are constructed into a large library of antibody fragments which contain reactivities against stanniocalcin to which the donor may or may not have been exposed (see e.g., U.S. Patent 5,885,793 incorporated herein in its entirety by reference).

[0536] *Rescue of the Library.* A library of scFvs is constructed from the RNA of human PBLs as described in WO92/01047. To rescue phage displaying antibody fragments, approximately 10^9 E. coli harbouring the phagemid are used to inoculate 50 ml of 2xTY containing 1% glucose and 100 ug/ml of ampicillin (2xTY-AMP-GLU) and grown to an O.D. of 0.8 with shaking. Five ml of this culture is used to inoculate 50 ml of 2xTY-AMP-GLU, 2×10^8 TU of delta gene 3 helper (M13 delta gene III, see WO92/01047) are added and the culture incubated at 37 degree C for 45 minutes without shaking and then at 37 degree C for 45 minutes with shaking. The culture is centrifuged at 4000 r.p.m. for 10 min. and the pellet resuspended in 2 liters of 2xTY containing 100 ug/ml ampicillin and 50 ug/ml kanamycin and grown overnight. Phage are prepared as described in WO92/01047.

[0537] M13 delta gene III is prepared as follows: M13 delta gene III helper phage does not encode gene III protein, hence the phage(mid) displaying antibody fragments have a greater avidity of binding to antigen. Infectious M13 delta gene III particles are made by growing the helper phage in cells harbouring a pUC19 derivative supplying the wild type gene III protein during phage morphogenesis. The culture is incubated for 1 hour at 37 degree C without shaking and then for a further hour at 37 degree C with shaking. Cells

are spun down (IEC-Centra 8, 4000 revs/min for 10 min), resuspended in 300 ml 2xTY broth containing 100 ug ampicillin/ml and 25 ug kanamycin/ml (2xTY-AMP-KAN) and grown overnight, shaking at 37° C. Phage particles are purified and concentrated from the culture medium by two PEG-precipitations (Sambrook et al., 1990), resuspended in 2 ml PBS and passed through a 0.45 um filter (Minisart NML; Sartorius) to give a final concentration of approximately 10^{13} transducing units/ml (ampicillin-resistant plasmids).

[0538] *Panning of the Library.* Immunotubes (Nunc) are coated overnight in PBS with 4 ml of either 100 ug/ml or 10 ug/ml of a polypeptide of the present invention. Tubes are blocked with 2% Marvel-PBS for 2 hours at 37 degree C and then washed 3 times in PBS. Approximately 10^{13} TU of phage is applied to the tube and incubated for 30 minutes at room temperature tumbling on an over and under turntable and then left to stand for another 1.5 hours. Tubes are washed 10 times with PBS 0.1% Tween-20 and 10 times with PBS. Phage are eluted by adding 1 ml of 100 mM triethylamine and rotating 15 minutes on an under and over turntable after which the solution is immediately neutralized with 0.5 ml of 1.0M Tris-HCl, pH 7.4. Phage are then used to infect 10 ml of mid-log E. coli TG1 by incubating eluted phage with bacteria for 30 minutes at 37 degree C. The E. coli are then plated on TYE plates containing 1% glucose and 100 ug/ml ampicillin. The resulting bacterial library is then rescued with delta gene 3 helper phage as described above to prepare phage for a subsequent round of selection. This process is then repeated for a total of 4 rounds of affinity purification with tube-washing increased to 20 times with PBS, 0.1% Tween-20 and 20 times with PBS for rounds 3 and 4.

[0539] *Characterization of Binders.* Eluted phage from the 3rd and 4th rounds of selection are used to infect E. coli HB 2151 and soluble scFv is produced (Marks, et al., 1991) from single colonies for assay. ELISAs are performed with microtitre plates coated with either 10 pg/ml of the polypeptide of the present invention in 50 mM bicarbonate pH 9.6. Plasmids positive in ELISA are further characterized by PCR fingerprinting (see e.g., WO92/01047) and then by sequencing.

Example 9: Screening Assay Identifying Neuronal Activity.

[0540] When cells undergo differentiation and proliferation, a group of genes are activated through many different signal transduction pathways. One of these genes, EGR1

(early growth response gene 1), is induced in various tissues and cell types upon activation. The promoter of EGR1 is responsible for such induction. Using the EGR1 promoter linked to reporter molecules, activation of cells can be assessed by stanniocalcin.

[0541] Particularly, the following protocol is used to assess neuronal activity in PC12 cell lines. PC12 cells (rat phenochromocytoma cells) are known to proliferate and/or differentiate by activation with a number of mitogens, such as TPA (tetradecanoyl phorbol acetate), NGF (nerve growth factor), and EGF (epidermal growth factor). The EGR1 gene expression is activated during this treatment. Thus, by stably transfecting PC12 cells with a construct containing an EGR promoter linked to SEAP reporter, activation of PC12 cells by stanniocalcin can be assessed.

[0542] The EGR/SEAP reporter construct can be assembled by the following protocol. The EGR-1 promoter sequence (-633 to +1)(Sakamoto K et al., *Oncogene* 6:867-871 (1991)) can be PCR amplified from human genomic DNA using the following primers:

[0543] 5' GCGCTCGAGGGATGACAGCGATAGAACCCCGG -3' (SEQ ID NO:11)

[0544] 5' GCGAAGCTTCGCGACTCCCCGGATCCGCCTC-3' (SEQ ID NO:12)

[0545] Using the GAS:SEAP/Neo vector produced in Example 13, EGR1 amplified product can then be inserted into this vector. Linearize the GAS:SEAP/Neo vector using restriction enzymes XhoI/HindIII, removing the GAS/SV40 stuffer. Restrict the EGR1 amplified product with these same enzymes. Ligate the vector and the EGR1 promoter.

[0546] To prepare 96 well-plates for cell culture, two mls of a coating solution (1:30 dilution of collagen type I (Upstate Biotech Inc. Cat#08-115) in 30% ethanol (filter sterilized)) is added per one 10 cm plate or 50 ml per well of the 96-well plate, and allowed to air dry for 2 hr.

[0547] PC12 cells are routinely grown in RPMI-1640 medium (Bio Whittaker) containing 10% horse serum (JRH BIOSCIENCES, Cat. # 12449-78P), 5% heat-inactivated fetal bovine serum (FBS) supplemented with 100 units/ml penicillin and 100 ug/ml streptomycin on a precoated 10 cm tissue culture dish. One to four split is done every three to four days. Cells are removed from the plates by scraping and resuspended with pipetting up and down for more than 15 times.

[0548] Transfect the EGR/SEAP/Neo construct into PC12 using the Lipofectamine protocol described in Example 12. EGR-SEAP/PC12 stable cells are obtained by growing the cells in 300 ug/ml G418. The G418-free medium is used for routine growth but every

one to two months, the cells should be re-grown in 300 ug/ml G418 for couple of passages.

[0549] To assay for neuronal activity, a 10 cm plate with cells around 70 to 80% confluent is screened by removing the old medium. Wash the cells once with PBS (Phosphate buffered saline). Then starve the cells in low serum medium (RPMI-1640 containing 1% horse serum and 0.5% FBS with antibiotics) overnight.

[0550] The next morning, remove the medium and wash the cells with PBS. Scrape off the cells from the plate, suspend the cells well in 2 ml low serum medium. Count the cell number and add more low serum medium to reach final cell density as 5×10^5 cells/ml.

[0551] Add 200 ul of the cell suspension to each well of 96-well plate (equivalent to 1×10^5 cells/well). Add 50 ul supernatant produced by Example 12, 37 degree C for 48 to 72 hr. As a positive control, a growth factor known to activate PC12 cells through EGR can be used, such as 50 ng/ul of Neuronal Growth Factor (NGF). Over fifty-fold induction of SEAP is typically seen in the positive control wells. SEAP assay the supernatant according to Example 5.

Example 10: Screening Assay Identifying Changes in Small Molecule Concentration and Membrane Permeability

[0552] Binding of a ligand to a receptor is known to alter intracellular levels of small molecules, such as calcium, potassium, sodium, and pH, as well as alter membrane potential. These alterations can be measured in an assay to identify supernatants which bind to receptors of a particular cell. Although the following protocol describes an assay for calcium, this protocol can easily be modified to detect changes in potassium, sodium, pH, membrane potential, or any other small molecule which is detectable by a fluorescent probe.

[0553] The following assay uses Fluorometric Imaging Plate Reader ("FLIPR") to measure changes in fluorescent molecules (Molecular Probes) that bind small molecules. Clearly, any fluorescent molecule detecting a small molecule can be used instead of the calcium fluorescent molecule, fluo-3, used here.

[0554] For adherent cells, seed the cells at 10,000 -20,000 cells/well in a Co-star black 96-well plate with clear bottom. The plate is incubated in a CO₂ incubator for 20 hours. The adherent cells are washed two times in Biotek washer with 200 ul of HBSS (Hank's Balanced Salt Solution) leaving 100 ul of buffer after the final wash.

[0555] A stock solution of 1 mg/ml fluo-3 is made in 10% pluronic acid DMSO. To load the cells with fluo-3, 50 ul of 12 ug/ml fluo-3 is added to each well. The plate is incubated at 37 degree C in a CO₂ incubator for 60 min. The plate is washed four times in the Biotek washer with HBSS leaving 100 ul of buffer.

[0556] For non-adherent cells, the cells are spun down from culture media. Cells are re-suspended to 2-5x10⁶ cells/ml with HBSS in a 50-ml conical tube. 4 ul of 1 mg/ml fluo-3 solution in 10% pluronic acid DMSO is added to each ml of cell suspension. The tube is then placed in a 37 degree C water bath for 30-60 min. The cells are washed twice with HBSS, resuspended to 1x10⁶ cells/ml, and dispensed into a microplate, 100 ul/well. The plate is centrifuged at 1000 rpm for 5 min. The plate is then washed once in Denley CellWash with 200 ul, followed by an aspiration step to 100 ul final volume.

[0557] For a non-cell based assay, each well contains a fluorescent molecule, such as fluo-3. The supernatant is added to the well, and a change in fluorescence is detected.

[0558] To measure the fluorescence of intracellular calcium, the FLIPR is set for the following parameters: (1) System gain is 300-800 mW; (2) Exposure time is 0.4 second; (3) Camera F/stop is F/2; (4) Excitation is 488 nm; (5) Emission is 530 nm; and (6) Sample addition is 50 ul. Increased emission at 530 nm indicates an extracellular signaling event caused by the a molecule, either stanniocalcin or a molecule induced by stanniocalcin, which has resulted in an increase in the intracellular Ca⁺⁺ concentration.

Example 11: Assay for SEAP Activity

[0559] As a reporter molecule for the assays described in Examples 14-17, SEAP activity is assayed using the Tropix Phospho-light Kit (Cat. BP-400) according to the following general procedure. The Tropix Phospho-light Kit supplies the Dilution, Assay, and Reaction Buffers used below.

[0560] Prime a dispenser with the 2.5x Dilution Buffer and dispense 15 ul of 2.5x dilution buffer into Optiplates containing 35 ul of a supernatant. Seal the plates with a

plastic sealer and incubate at 65 degree C for 30 min. Separate the Optiplates to avoid uneven heating.

[0561] Cool the samples to room temperature for 15 minutes. Empty the dispenser and prime with the Assay Buffer. Add 50 ml Assay Buffer and incubate at room temperature 5 min. Empty the dispenser and prime with the Reaction Buffer (see the table below). Add 50 ul Reaction Buffer and incubate at room temperature for 20 minutes. Since the intensity of the chemiluminescent signal is time dependent, and it takes about 10 minutes to read 5 plates on luminometer, one should treat 5 plates at each time and start the second set 10 minutes later.

[0562] Read the relative light unit in the luminometer. Set H12 as blank, and print the results. An increase in chemiluminescence indicates reporter activity.

Reaction Buffer Formulation:

# of plates	Rxn buffer diluent (ml)	CSPD (ml)
10	60	3
11	65	3.25
12	70	3.5
13	75	3.75
14	80	4
15	85	4.25
16	90	4.5
17	95	4.75
18	100	5
19	105	5.25
20	110	5.5
21	115	5.75
22	120	6
23	125	6.25
24	130	6.5
25	135	6.75
26	140	7
27	145	7.25
28	150	7.5
29	155	7.75
30	160	8

# of plates	Rxn buffer diluent (ml)	CSPD (ml)
31	165	8.25
32	170	8.5
33	175	8.75
34	180	9
35	185	9.25
36	190	9.5
37	195	9.75
38	200	10
39	205	10.25
40	210	10.5
41	215	10.75
42	220	11
43	225	11.25
44	230	11.5
45	235	11.75
46	240	12
47	245	12.25
48	250	12.5
49	255	12.75
50	260	13

***Example 12: Method of Detecting Abnormal Levels
of Stanniocalcin in a Biological Sample***

[0563] Stanniocalcin polypeptides can be detected in a biological sample, and if an increased or decreased level of stanniocalcin is detected, this polypeptide is a marker for a particular phenotype. Methods of detection are numerous, and thus, it is understood that one skilled in the art can modify the following assay to fit their particular needs.

[0564] For example, antibody-sandwich ELISAs are used to detect stanniocalcin in a sample, preferably a biological sample. Wells of a microtiter plate are coated with specific antibodies to stanniocalcin, at a final concentration of 0.2 to 10 ug/ml. The antibodies are either monoclonal or polyclonal and are produced by the method described in Example 11. The wells are blocked so that non-specific binding of stanniocalcin to the well is reduced.

[0565] The coated wells are then incubated for > 2 hours at RT with a sample containing stanniocalcin. Preferably, serial dilutions of the sample should be used to validate results. The plates are then washed three times with deionized or distilled water to remove unbounded stanniocalcin.

[0566] Next, 50 ul of specific antibody-alkaline phosphatase conjugate, at a concentration of 25-400 ng, is added and incubated for 2 hours at room temperature. The plates are again washed three times with deionized or distilled water to remove unbounded conjugate.

[0567] Add 75 ul of 4-methylumbelliferyl phosphate (MUP) or p-nitrophenyl phosphate (NPP) substrate solution to each well and incubate 1 hour at room temperature. Measure the reaction by a microtiter plate reader. Prepare a standard curve, using serial dilutions of a control sample, and plot stanniocalcin polypeptide concentration on the X-axis (log scale) and fluorescence or absorbance of the Y-axis (linear scale). Interpolate the concentration of the stanniocalcin in the sample using the standard curve.

Example 13: Formulating a Polypeptide

[0568] The invention also provides methods of treatment and/or prevention of diseases or disorders (such as, for example, any one or more of the diseases or disorders disclosed herein) by administration to a subject of an effective amount of a Therapeutic. By therapeutic is meant polynucleotides or polypeptides of the invention (including fragments and variants), agonists or antagonists thereof, and/or antibodies thereto, in combination with a pharmaceutically acceptable carrier type (e.g., a sterile carrier).

[0569] The Therapeutic will be formulated and dosed in a fashion consistent with good medical practice, taking into account the clinical condition of the individual patient (especially the side effects of treatment with the Therapeutic alone), the site of delivery, the method of administration, the scheduling of administration, and other factors known to practitioners. The “effective amount” for purposes herein is thus determined by such considerations.

[0570] As a general proposition, the total pharmaceutically effective amount of the Therapeutic administered parenterally per dose will be in the range of about 1ug/kg/day to 10 mg/kg/day of patient body weight, although, as noted above, this will be subject to

therapeutic discretion. More preferably, this dose is at least 0.01 mg/kg/day, and most preferably for humans between about 0.01 and 1 mg/kg/day for the hormone. If given continuously, the Therapeutic is typically administered at a dose rate of about 1 ug/kg/hour to about 50 ug/kg/hour, either by 1-4 injections per day or by continuous subcutaneous infusions, for example, using a mini-pump. An intravenous bag solution may also be employed. The length of treatment needed to observe changes and the interval following treatment for responses to occur appears to vary depending on the desired effect.

[0571] Therapeutics can be administered orally, rectally, parenterally, intracisternally, intravaginally, intraperitoneally, topically (as by powders, ointments, gels, drops or transdermal patch), buccally, or as an oral or nasal spray. "Pharmaceutically acceptable carrier" refers to a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any. The term "parenteral" as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion.

[0572] Therapeutics of the invention are also suitably administered by sustained-release systems. Suitable examples of sustained-release Therapeutics are administered orally, rectally, parenterally, intracisternally, intravaginally, intraperitoneally, topically (as by powders, ointments, gels, drops or transdermal patch), buccally, or as an oral or nasal spray. "Pharmaceutically acceptable carrier" refers to a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. The term "parenteral" as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion.

[0573] Therapeutics of the invention are also suitably administered by sustained-release systems. Suitable examples of sustained-release Therapeutics include suitable polymeric materials (such as, for example, semi-permeable polymer matrices in the form of shaped articles, e.g., films, or microcapsules), suitable hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, and sparingly soluble derivatives (such as, for example, a sparingly soluble salt).

[0574] Sustained-release matrices include polylactides (U.S. Pat. No. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma-ethyl-L-glutamate (Sidman et al., Biopolymers 22:547-556 (1983)), poly (2- hydroxyethyl methacrylate) (Langer et al., J. Biomed. Mater. Res. 15:167-277 (1981), and Langer, Chem. Tech. 12:98-105 (1982)),

ethylene vinyl acetate (Langer et al., Id.) or poly-D- (-)-3-hydroxybutyric acid (EP 133,988).

[0575] Sustained-release Therapeutics also include liposomally entrapped Therapeutics of the invention (*see generally*, Langer, *Science* 249:1527-1533 (1990); Treat et al., in *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 317 -327 and 353-365 (1989)). Liposomes containing the Therapeutic are prepared by methods known per se: DE 3,218,121; Epstein et al., Proc. Natl. Acad. Sci. (USA) 82:3688-3692 (1985); Hwang et al., Proc. Natl. Acad. Sci.(USA) 77:4030-4034 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese Pat. Appl. 83-118008; U.S. Pat. Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily, the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. percent cholesterol, the selected proportion being adjusted for the optimal Therapeutic.

[0576] In yet an additional embodiment, the Therapeutics of the invention are delivered by way of a pump (*see* Langer, *supra*; Sefton, CRC Crit. Ref. Biomed. Eng. 14:201 (1987); Buchwald et al., Surgery 88:507 (1980); Saudek et al., N. Engl. J. Med. 321:574 (1989)).

[0577] Other controlled release systems are discussed in the review by Langer (*Science* 249:1527-1533 (1990)).

[0578] For parenteral administration, in one embodiment, the Therapeutic is formulated generally by mixing it at the desired degree of purity, in a unit dosage injectable form (solution, suspension, or emulsion), with a pharmaceutically acceptable carrier, i.e., one that is non-toxic to recipients at the dosages and concentrations employed and is compatible with other ingredients of the formulation. For example, the formulation preferably does not include oxidizing agents and other compounds that are known to be deleterious to the Therapeutic.

[0579] Generally, the formulations are prepared by contacting the Therapeutic uniformly and intimately with liquid carriers or finely divided solid carriers or both. Then, if necessary, the product is shaped into the desired formulation. Preferably the carrier is a parenteral carrier, more preferably a solution that is isotonic with the blood of the recipient. Examples of such carrier vehicles include water, saline, Ringer's solution, and

dextrose solution. Non-aqueous vehicles such as fixed oils and ethyl oleate are also useful herein, as well as liposomes.

[0580] The carrier suitably contains minor amounts of additives such as substances that enhance isotonicity and chemical stability. Such materials are non-toxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, succinate, acetic acid, and other organic acids or their salts; antioxidants such as ascorbic acid; low molecular weight (less than about ten residues) polypeptides, e.g., polyarginine or tripeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids, such as glycine, glutamic acid, aspartic acid, or arginine; monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; counterions such as sodium; and/or nonionic surfactants such as polysorbates, poloxamers, or PEG.

[0581] The Therapeutic is typically formulated in such vehicles at a concentration of about 0.1 mg/ml to 100 mg/ml, preferably 1-10 mg/ml, at a pH of about 3 to 8. It will be understood that the use of certain of the foregoing excipients, carriers, or stabilizers will result in the formation of polypeptide salts.

[0582] Any pharmaceutical used for therapeutic administration can be sterile. Sterility is readily accomplished by filtration through sterile filtration membranes (e.g., 0.2 micron membranes). Therapeutics generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

[0583] Therapeutics ordinarily will be stored in unit or multi-dose containers, for example, sealed ampoules or vials, as an aqueous solution or as a lyophilized formulation for reconstitution. As an example of a lyophilized formulation, 10-ml vials are filled with 5 ml of sterile-filtered 1% (w/v) aqueous Therapeutic solution, and the resulting mixture is lyophilized. The infusion solution is prepared by reconstituting the lyophilized Therapeutic using bacteriostatic Water-for-Injection.

[0584] The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the Therapeutics of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or

biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the Therapeutics may be employed in conjunction with other therapeutic compounds.

[0585] The Therapeutics of the invention may be administered alone or in combination with adjuvants. Adjuvants that may be administered with the Therapeutics of the invention include, but are not limited to, alum, alum plus deoxycholate (ImmunoAg), MTP-PE (Biocine Corp.), QS21 (Genentech, Inc.), BCG (e.g., THERACYS®), MPL and nonviable preparations of *Corynebacterium parvum*. In a specific embodiment, Therapeutics of the invention are administered in combination with alum. In another specific embodiment, Therapeutics of the invention are administered in combination with QS-21. Further adjuvants that may be administered with the Therapeutics of the invention include, but are not limited to, Monophosphoryl lipid immunomodulator, AdjuVax 100a, QS-21, QS-18, CRL1005, Aluminum salts, MF-59, and Virosomal adjuvant technology. Vaccines that may be administered with the Therapeutics of the invention include, but are not limited to, vaccines directed toward protection against MMR (measles, mumps, rubella), polio, varicella, tetanus/diphtheria, hepatitis A, hepatitis B, haemophilus influenzae B, whooping cough, pneumonia, influenza, Lyme's Disease, rotavirus, cholera, yellow fever, Japanese encephalitis, poliomyelitis, rabies, typhoid fever, and pertussis. Combinations may be administered either concomitantly, e.g., as an admixture, separately but simultaneously or concurrently; or sequentially. This includes presentations in which the combined agents are administered together as a therapeutic mixture, and also procedures in which the combined agents are administered separately but simultaneously, e.g., as through separate intravenous lines into the same individual. Administration "in combination" further includes the separate administration of one of the compounds or agents given first, followed by the second.

[0586] The Therapeutics of the invention may be administered alone or in combination with other therapeutic agents. Therapeutic agents that may be administered in combination with the Therapeutics of the invention, include but not limited to, chemotherapeutic agents, antibiotics, steroidal and non-steroidal anti-inflammatories, conventional immunotherapeutic agents, and/or therapeutic treatments described below. Combinations may be administered either concomitantly, e.g., as an admixture, separately but simultaneously or concurrently; or sequentially. This includes presentations in which

the combined agents are administered together as a therapeutic mixture, and also procedures in which the combined agents are administered separately but simultaneously, e.g., as through separate intravenous lines into the same individual. Administration "in combination" further includes the separate administration of one of the compounds or agents given first, followed by the second.

[0587] In one embodiment, the Therapeutics of the invention are administered in combination with an anticoagulant. Anticoagulants that may be administered with the compositions of the invention include, but are not limited to, heparin, low molecular weight heparin, warfarin sodium (e.g., COUMADIN®), dicumarol, 4-hydroxycoumarin, anisindione (e.g., MIRADON™), acenocoumarol (e.g., nicoumalone, SINTHROME™), indan-1,3-dione, phenprocoumon (e.g., MARCUMAR™), ethyl biscoumacetate (e.g., TROMEXAN™), and aspirin. In a specific embodiment, compositions of the invention are administered in combination with heparin and/or warfarin. In another specific embodiment, compositions of the invention are administered in combination with warfarin. In another specific embodiment, compositions of the invention are administered in combination with warfarin and aspirin. In another specific embodiment, compositions of the invention are administered in combination with heparin. In another specific embodiment, compositions of the invention are administered in combination with heparin and aspirin.

[0588] In another embodiment, the Therapeutics of the invention are administered in combination with thrombolytic drugs. Thrombolytic drugs that may be administered with the compositions of the invention include, but are not limited to, plasminogen, lys-plasminogen, alpha2-antiplasmin, streptokinase (e.g., KABIKINASE™), antiresplace (e.g., EMINASE™), tissue plasminogen activator (t-PA, altevase, ACTIVASE™), urokinase (e.g., ABBOKINASE™), sauruplase, (Prourokinase, single chain urokinase), and aminocaproic acid (e.g., AMICAR™). In a specific embodiment, compositions of the invention are administered in combination with tissue plasminogen activator and aspirin.

[0589] In another embodiment, the Therapeutics of the invention are administered in combination with antiplatelet drugs. Antiplatelet drugs that may be administered with the compositions of the invention include, but are not limited to, aspirin, dipyridamole (e.g., PERSANTINE™), and ticlopidine (e.g., TICLID™).

[0590] In specific embodiments, the use of anti-coagulants, thrombolytic and/or antiplatelet drugs in combination with Therapeutics of the invention is contemplated for the prevention, diagnosis, and/or treatment of thrombosis, arterial thrombosis, venous thrombosis, thromboembolism, pulmonary embolism, atherosclerosis, myocardial infarction, transient ischemic attack, unstable angina. In specific embodiments, the use of anticoagulants, thrombolytic drugs and/or antiplatelet drugs in combination with Therapeutics of the invention is contemplated for the prevention of occlusion of saphenous grafts, for reducing the risk of periprocedural thrombosis as might accompany angioplasty procedures, for reducing the risk of stroke in patients with atrial fibrillation including nonrheumatic atrial fibrillation, for reducing the risk of embolism associated with mechanical heart valves and or mitral valves disease. Other uses for the therapeutics of the invention, alone or in combination with antiplatelet, anticoagulant, and/or thrombolytic drugs, include, but are not limited to, the prevention of occlusions in extracorporeal devices (e.g., intravascular canulas, vascular access shunts in hemodialysis patients, hemodialysis machines, and cardiopulmonary bypass machines).

[0591] In certain embodiments, Therapeutics of the invention are administered in combination with antiretroviral agents, nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and/or protease inhibitors (PIs). NRTIs that may be administered in combination with the Therapeutics of the invention, include, but are not limited to, RETROVIR™ (zidovudine/AZT), VIDEX™ (didanosine/ddI), HIVID™ (zalcitabine/ddC), ZERIT™ (stavudine/d4T), EPIVIR™ (lamivudine/3TC), and COMBIVIR™ (zidovudine/lamivudine). NNRTIs that may be administered in combination with the Therapeutics of the invention, include, but are not limited to, VIRAMUNE™ (nevirapine), RESCRIPTOR™ (delavirdine), and SUSTIVA™ (efavirenz). Protease inhibitors that may be administered in combination with the Therapeutics of the invention, include, but are not limited to, CRIXIVAN™ (indinavir), NORVIR™ (ritonavir), INVIRASE™ (saquinavir), and VIRACEPT™ (nelfinavir). In a specific embodiment, antiretroviral agents, nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, and/or protease inhibitors may be used in any combination with Therapeutics of the invention to treat AIDS and/or to prevent or treat HIV infection.

[0592] Additional NRTIs include LODENOSINE™ (F-ddA; an acid-stable adenosine NRTI; Triangle/Abbott); COVIRACIL™ (emtricitabine/FTC; structurally related to lamivudine (3TC) but with 3- to 10-fold greater activity *in vitro*; Triangle/Abbott); dOTC (BCH-10652, also structurally related to lamivudine but retains activity against a substantial proportion of lamivudine-resistant isolates; Biochem Pharma); Adefovir (refused approval for anti-HIV therapy by FDA; Gilead Sciences); PREVEON® (Adefovir Dipivoxil, the active prodrug of adefovir; its active form is PMEA-pp); TENOFOVIR™ (bis-POC PMPA, a PMPA prodrug; Gilead); DAPD/DXG (active metabolite of DAPD; Triangle/Abbott); D-D4FC (related to 3TC, with activity against AZT/3TC-resistant virus); GW420867X (Glaxo Wellcome); ZIAGEN™ (abacavir/159U89; Glaxo Wellcome Inc.); CS-87 (3'-azido-2',3'-dideoxyuridine; WO 99/66936); and S-acyl-2-thioethyl (SATE)-bearing prodrug forms of β -L-FD4C and β -L-FddC (WO 98/17281).

[0593] Additional NNRTIs include COACTINON™ (Emivirine/MKC-442, potent NNRTI of the HEPT class; Triangle/Abbott); CAPRAVIRINE™ (AG-1549/S-1153, a next generation NNRTI with activity against viruses containing the K103N mutation; Agouron); PNU-142721 (has 20- to 50-fold greater activity than its predecessor delavirdine and is active against K103N mutants; Pharmacia & Upjohn); DPC-961 and DPC-963 (second-generation derivatives of efavirenz, designed to be active against viruses with the K103N mutation; DuPont); GW-420867X (has 25-fold greater activity than HBY097 and is active against K103N mutants; Glaxo Wellcome); CALANOLIDE A (naturally occurring agent from the latex tree; active against viruses containing either or both the Y181C and K103N mutations); and Propolis (WO 99/49830).

[0594] Additional protease inhibitors include LOPINAVIR™ (ABT378/r; Abbott Laboratories); BMS-232632 (an azapeptide; Bristol-Myers Squibb); TIPRANAVIR™ (PNU-140690, a non-peptic dihydropyrone; Pharmacia & Upjohn); PD-178390 (a nonpeptidic dihydropyrone; Parke-Davis); BMS 232632 (an azapeptide; Bristol-Myers Squibb); L-756,423 (an indinavir analog; Merck); DMP-450 (a cyclic urea compound; Avid & DuPont); AG-1776 (a peptidomimetic with *in vitro* activity against protease inhibitor-resistant viruses; Agouron); VX-175/GW-433908 (phosphate prodrug of amprenavir; Vertex & Glaxo Wellcome); CGP61755 (Ciba); and AGENERASE™ (amprenavir; Glaxo Wellcome Inc.).

[0595] Additional antiretroviral agents include fusion inhibitors/gp41 binders. Fusion inhibitors/gp41 binders include T-20 (a peptide from residues 643-678 of the HIV gp41 transmembrane protein ectodomain which binds to gp41 in its resting state and prevents transformation to the fusogenic state; Trimeris) and T-1249 (a second-generation fusion inhibitor; Trimeris).

[0596] Additional antiretroviral agents include fusion inhibitors/chemokine receptor antagonists. Fusion inhibitors/chemokine receptor antagonists include CXCR4 antagonists such as AMD 3100 (a bicyclam), SDF-1 and its analogs, and ALX40-4C (a cationic peptide), T22 (an 18 amino acid peptide; Trimeris) and the T22 analogs T134 and T140; CCR5 antagonists such as RANTES (9-68), AOP-RANTES, NNY-RANTES, and TAK-779; and CCR5/CXCR4 antagonists such as NSC 651016 (a distamycin analog). Also included are CCR2B, CCR3, and CCR6 antagonists. Chemokine receptor agonists such as RANTES, SDF-1, MIP-1 α , MIP-1 β , etc., may also inhibit fusion.

[0597] Additional antiretroviral agents include integrase inhibitors. Integrase inhibitors include dicaffeoylquinic (DFQA) acids; L-chicoric acid (a dicaffeoyltartaric (DCTA) acid); quinalizarin (QLC) and related anthraquinones; ZINTEVIR™ (AR 177, an oligonucleotide that probably acts at cell surface rather than being a true integrase inhibitor; Arondex); and naphthols such as those disclosed in WO 98/50347.

[0598] Additional antiretroviral agents include hydroxyurea-like compounds such as BCX-34 (a purine nucleoside phosphorylase inhibitor; Biocryst); ribonucleotide reductase inhibitors such as DIDOX™ (Molecules for Health); inosine monophosphate dehydrogenase (IMPDH) inhibitors such as VX-497 (Vertex); and mycopholic acids such as CellCept (mycophenolate mofetil; Roche).

[0599] Additional antiretroviral agents include inhibitors of viral integrase, inhibitors of viral genome nuclear translocation such as arylene bis(methylketone) compounds; inhibitors of HIV entry such as AOP-RANTES, NNY-RANTES, RANTES-IgG fusion protein, soluble complexes of RANTES and glycosaminoglycans (GAG), and AMD-3100; nucleocapsid zinc finger inhibitors such as dithiane compounds; targets of HIV Tat and Rev; and pharmacoenhancers such as ABT-378.

[0600] Other antiretroviral therapies and adjunct therapies include cytokines and lymphokines such as MIP-1 α , MIP-1 β , SDF-1 α , IL-2, PROLEUKIN™ (aldesleukin/L2-7001; Chiron), IL-4, IL-10, IL-12, and IL-13; interferons such as IFN- α 2a; antagonists of

TNFs, NFκB, GM-CSF, M-CSF, and IL-10; agents that modulate immune activation such as cyclosporin and prednisone; vaccines such as Remune™ (HIV Immunogen), APL 400-003 (Apollon), recombinant gp120 and fragments, bivalent (B/E) recombinant envelope glycoprotein, rgp120CM235, MN rgp120, SF-2 rgp120, gp120/soluble CD4 complex, Delta JR-FL protein, branched synthetic peptide derived from discontinuous gp120 C3/C4 domain, fusion-competent immunogens, and Gag, Pol, Nef, and Tat vaccines; gene-based therapies such as genetic suppressor elements (GSEs; WO 98/54366), and intrakines (genetically modified CC chemokines targeted to the ER to block surface expression of newly synthesized CCR5 (Yang *et al.*, *PNAS* 94:11567-72 (1997); Chen *et al.*, *Nat. Med.* 3:1110-16 (1997)); antibodies such as the anti-CXCR4 antibody 12G5, the anti-CCR5 antibodies 2D7, 5C7, PA8, PA9, PA10, PA11, PA12, and PA14, the anti-CD4 antibodies Q4120 and RPA-T4, the anti-CCR3 antibody 7B11, the anti-gp120 antibodies 17b, 48d, 447-52D, 257-D, 268-D and 50.1, anti-Tat antibodies, anti-TNF-α antibodies, and monoclonal antibody 33A; aryl hydrocarbon (AH) receptor agonists and antagonists such as TCDD, 3,3',4,4',5-pentachlorobiphenyl, 3,3',4,4'-tetrachlorobiphenyl, and α-naphthoflavone (WO 98/30213); and antioxidants such as γ-L-glutamyl-L-cysteine ethyl ester (γ-GCE; WO 99/56764).

[0601] In a further embodiment, the Therapeutics of the invention are administered in combination with an antiviral agent. Antiviral agents that may be administered with the Therapeutics of the invention include, but are not limited to, acyclovir, ribavirin, amantadine, and remantidine.

[0602] In other embodiments, Therapeutics of the invention may be administered in combination with anti-opportunistic infection agents. Anti-opportunistic agents that may be administered in combination with the Therapeutics of the invention, include, but are not limited to, TRIMETHOPRIM-SULFAMETHOXAZOLE™, DAPSONE™, PENTAMIDINE™, ATOVAQUONE™, ISONIAZID™, RIFAMPIN™, PYRAZINAMIDE™, ETHAMBUTOL™, RIFABUTIN™, CLARITHROMYCIN™, AZITHROMYCIN™, GANCICLOVIR™, FOSCARNET™, CIDOFOVIR™, FLUCONAZOLE™, ITRACONAZOLE™, KETOCONAZOLE™, ACYCLOVIR™, FAMCICLOVIR™, PYRIMETHAMINE™, LEUCOVORIN™, NEUPOGEN™ (filgrastim/G-CSF), and LEUKINE™ (sargramostim/GM-CSF). In a specific embodiment,

Therapeutics of the invention are used in any combination with TRIMETHOPRIM-SULFAMETHOXAZOLE™, DAPSONE™, PENTAMIDINE™, and/or ATOVAQUONE™ to prophylactically treat or prevent an opportunistic *Pneumocystis carinii* pneumonia infection. In another specific embodiment, Therapeutics of the invention are used in any combination with ISONIAZID™, RIFAMPIN™, PYRAZINAMIDE™, and/or ETHAMBUTOL™ to prophylactically treat or prevent an opportunistic *Mycobacterium avium* complex infection. In another specific embodiment, Therapeutics of the invention are used in any combination with RIFABUTIN™, CLARITHROMYCIN™, and/or AZITHROMYCIN™ to prophylactically treat or prevent an opportunistic *Mycobacterium tuberculosis* infection. In another specific embodiment, Therapeutics of the invention are used in any combination with GANCICLOVIR™, FOSCARNET™, and/or CIDOFOVIR™ to prophylactically treat or prevent an opportunistic cytomegalovirus infection. In another specific embodiment, Therapeutics of the invention are used in any combination with FLUCONAZOLE™, ITRACONAZOLE™, and/or KETOCONAZOLE™ to prophylactically treat or prevent an opportunistic fungal infection. In another specific embodiment, Therapeutics of the invention are used in any combination with ACYCLOVIR™ and/or FAMCICOLVIR™ to prophylactically treat or prevent an opportunistic herpes simplex virus type I and/or type II infection. In another specific embodiment, Therapeutics of the invention are used in any combination with PYRIMETHAMINE™ and/or LEUCOVORIN™ to prophylactically treat or prevent an opportunistic *Toxoplasma gondii* infection. In another specific embodiment, Therapeutics of the invention are used in any combination with LEUCOVORIN™ and/or NEUPOGEN™ to prophylactically treat or prevent an opportunistic bacterial infection.

[0603] In a further embodiment, the Therapeutics of the invention are administered in combination with an antibiotic agent. Antibiotic agents that may be administered with the Therapeutics of the invention include, but are not limited to, amoxicillin, beta-lactamases, aminoglycosides, beta-lactam (glycopeptide), beta-lactamases, Clindamycin, chloramphenicol, cephalosporins, ciprofloxacin, erythromycin, fluoroquinolones, macrolides, metronidazole, penicillins, quinolones, rapamycin, rifampin, streptomycin, sulfonamide, tetracyclines, trimethoprim, trimethoprim-sulfamethoxazole, and vancomycin.

[0604] In other embodiments, the Therapeutics of the invention are administered in combination with immunostimulants. Immunostimulants that may be administered in combination with the Therapeutics of the invention include, but are not limited to, levamisole (e.g., ERGAMISOL™), isoprinosine (e.g. INOSIPLEX™), interferons (e.g. interferon alpha), and interleukins (e.g., IL-2).

[0605] In other embodiments, Therapeutics of the invention are administered in combination with immunosuppressive agents. Immunosuppressive agents that may be administered in combination with the Therapeutics of the invention include, but are not limited to, steroids, cyclosporine, cyclosporine analogs, cyclophosphamide methylprednisone, prednisone, azathioprine, FK-506, 15-deoxyspergualin, and other immunosuppressive agents that act by suppressing the function of responding T cells. Other immunosuppressive agents that may be administered in combination with the Therapeutics of the invention include, but are not limited to, prednisolone, methotrexate, thalidomide, methoxsalen, rapamycin, leflunomide, mizoribine (BREDININ™), brequinar, deoxyspergualin, and azaspirane (SKF 105685), ORTHOCLONE OKT® 3 (muromonab-CD3), SANDIMMUNE™, NEORAL™, SANGDYA™ (cyclosporine), PROGRAF® (FK506, tacrolimus), CELLCEPT® (mycophenolate mofetil, of which the active metabolite is mycophenolic acid), IMURAN™ (azathioprine), glucocorticosteroids, adrenocortical steroids such as DELTASONE™ (prednisone) and HYDELTRASOL™ (prednisolone), FOLEX™ and MEXATE™ (methotrexate), OXSORALEN-ULTRA™ (methoxsalen) and RAPAMUNE™ (sirolimus). In a specific embodiment, immunosuppressants may be used to prevent rejection of organ or bone marrow transplantation.

[0606] In an additional embodiment, Therapeutics of the invention are administered alone or in combination with one or more intravenous immune globulin preparations. Intravenous immune globulin preparations that may be administered with the Therapeutics of the invention include, but not limited to, GAMMAR™, IVEEGAM™, SANDOGLOBULIN™, GAMMAGARD S/D™, ATGAM™ (antithymocyte globulin), and GAMIMUNE™. In a specific embodiment, Therapeutics of the invention are administered in combination with intravenous immune globulin preparations in transplantation therapy (e.g., bone marrow transplant).

[0607] In certain embodiments, the Therapeutics of the invention are administered alone or in combination with an anti-inflammatory agent. Anti-inflammatory agents that may be administered with the Therapeutics of the invention include, but are not limited to, corticosteroids (e.g. betamethasone, budesonide, cortisone, dexamethasone, hydrocortisone, methylprednisolone, prednisolone, prednisone, and triamcinolone), nonsteroidal anti-inflammatory drugs (e.g., diclofenac, diflunisal, etodolac, fenoprofen, floctafenine, flurbiprofen, ibuprofen, indomethacin, ketoprofen, meclofenamate, mefenamic acid, meloxicam, nabumetone, naproxen, oxaprozin, phenylbutazone, piroxicam, sulindac, tenoxicam, tiaprofenic acid, and tolmetin.), as well as antihistamines, aminoarylcarboxylic acid derivatives, arylacetic acid derivatives, arylbutyric acid derivatives, arylcarboxylic acids, arylpropionic acid derivatives, pyrazoles, pyrazolones, salicylic acid derivatives, thiazinecarboxamides, e-acetamidocaproic acid, S-adenosylmethionine, 3-amino-4-hydroxybutyric acid, amixetrine, bendazac, benzydamine, bucolome, difenpiramide, ditazol, emorfazone, guaiazulene, nabumetone, nimesulide, orgotein, oxaceprol, paranyline, perisoxal, pifoxime, proquazone, proxazole, and tenidap.

[0608] In an additional embodiment, the compositions of the invention are administered alone or in combination with an anti-angiogenic agent. Anti-angiogenic agents that may be administered with the compositions of the invention include, but are not limited to, Angiostatin (Entremed, Rockville, MD), Troponin-1 (Boston Life Sciences, Boston, MA), anti-Invasive Factor, retinoic acid and derivatives thereof, paclitaxel (Taxol), Suramin, Tissue Inhibitor of Metalloproteinase-1, Tissue Inhibitor of Metalloproteinase-2, VEGI, Plasminogen Activator Inhibitor-1, Plasminogen Activator Inhibitor-2, and various forms of the lighter “d group” transition metals.

[0609] Lighter “d group” transition metals include, for example, vanadium, molybdenum, tungsten, titanium, niobium, and tantalum species. Such transition metal species may form transition metal complexes. Suitable complexes of the above-mentioned transition metal species include oxo transition metal complexes.

[0610] Representative examples of vanadium complexes include oxo vanadium complexes such as vanadate and vanadyl complexes. Suitable vanadate complexes include metavanadate and orthovanadate complexes such as, for example, ammonium metavanadate, sodium metavanadate, and sodium orthovanadate. Suitable vanadyl

complexes include, for example, vanadyl acetylacetonate and vanadyl sulfate including vanadyl sulfate hydrates such as vanadyl sulfate mono- and trihydrates.

[0611] Representative examples of tungsten and molybdenum complexes also include oxo complexes. Suitable oxo tungsten complexes include tungstate and tungsten oxide complexes. Suitable tungstate complexes include ammonium tungstate, calcium tungstate, sodium tungstate dihydrate, and tungstic acid. Suitable tungsten oxides include tungsten (IV) oxide and tungsten (VI) oxide. Suitable oxo molybdenum complexes include molybdate, molybdenum oxide, and molybdenyl complexes. Suitable molybdate complexes include ammonium molybdate and its hydrates, sodium molybdate and its hydrates, and potassium molybdate and its hydrates. Suitable molybdenum oxides include molybdenum (VI) oxide, molybdenum (VI) oxide, and molybdic acid. Suitable molybdenyl complexes include, for example, molybdenyl acetylacetonate. Other suitable tungsten and molybdenum complexes include hydroxo derivatives derived from, for example, glycerol, tartaric acid, and sugars.

[0612] A wide variety of other anti-angiogenic factors may also be utilized within the context of the present invention. Representative examples include, but are not limited to, platelet factor 4; protamine sulphate; sulphated chitin derivatives (prepared from queen crab shells), (Murata et al., *Cancer Res.* 51:22-26, (1991)); Sulphated Polysaccharide Peptidoglycan Complex (SP- PG) (the function of this compound may be enhanced by the presence of steroids such as estrogen, and tamoxifen citrate); Staurosporine; modulators of matrix metabolism, including for example, proline analogs, cishydroxyproline, d,L-3,4-dehydroproline, Thiaproline, alpha,alpha-dipyridyl, aminopropionitrile fumarate; 4-propyl-5-(4-pyridinyl)-2(3H)-oxazolone; Methotrexate; Mitoxantrone; Heparin; Interferons; 2 Macroglobulin-serum; ChIMP-3 (Pavloff et al., *J. Bio. Chem.* 267:17321-17326, (1992)); Chymostatin (Tomkinson et al., *Biochem J.* 286:475-480, (1992)); Cyclodextrin Tetradecasulfate; Eponemycin; Camptothecin; Fumagillin (Ingber et al., *Nature* 348:555-557, (1990)); Gold Sodium Thiomalate ("GST"; Matsubara and Ziff, *J. Clin. Invest.* 79:1440-1446, (1987)); anticollagenase-serum; alpha2-antiplasmin (Holmes et al., *J. Biol. Chem.* 262(4):1659-1664, (1987)); Bisantrene (National Cancer Institute); Lobenzarit disodium (N-(2)-carboxyphenyl-4- chloroanthronilic acid disodium or "CCA"; (Takeuchi et al., *Agents Actions* 36:312-316, (1992)); and metalloproteinase inhibitors such as BB94.

[0613] Additional anti-angiogenic factors that may also be utilized within the context of the present invention include Thalidomide, (Celgene, Warren, NJ); Angiostatic steroid; AGM-1470 (H. Brem and J. Folkman *J Pediatr. Surg.* 28:445-51 (1993)); an integrin alpha v beta 3 antagonist (C. Storgard et al., *J Clin. Invest.* 103:47-54 (1999)); carboxynaminolmidazole; Carboxyamidotriazole (CAI) (National Cancer Institute, Bethesda, MD); Conbretastatin A-4 (CA4P) (OXiGENE, Boston, MA); Squalamine (Magainin Pharmaceuticals, Plymouth Meeting, PA); TNP-470, (Tap Pharmaceuticals, Deerfield, IL); ZD-0101 AstraZeneca (London, UK); APRA (CT2584); Benefin, Byrostatin-1 (SC339555); CGP-41251 (PKC 412); CM101; Dexrazoxane (ICRF187); DMXAA; Endostatin; Flavopridiol; Genestein; GTE; ImmTher; Iressa (ZD1839); Octreotide (Somatostatin); Panretin; Penacillamine; Photopoint; PI-88; Prinomastat (AG-3340) Purlytin; Suradista (FCE26644); Tamoxifen (Nolvadex); Tazarotene; Tetrathiomolybdate; Xeloda (Capecitabine); and 5-Fluorouracil.

[0614] Anti-angiogenic agents that may be administered in combination with the compounds of the invention may work through a variety of mechanisms including, but not limited to, inhibiting proteolysis of the extracellular matrix, blocking the function of endothelial cell-extracellular matrix adhesion molecules, by antagonizing the function of angiogenesis inducers such as growth factors, and inhibiting integrin receptors expressed on proliferating endothelial cells. Examples of anti-angiogenic inhibitors that interfere with extracellular matrix proteolysis and which may be administered in combination with the compositions of the invention include, but are not limited to, AG-3340 (Agouron, La Jolla, CA), BAY-12-9566 (Bayer, West Haven, CT), BMS-275291 (Bristol Myers Squibb, Princeton, NJ), CGS-27032A (Novartis, East Hanover, NJ), Marimastat (British Biotech, Oxford, UK), and Metastat (Aeterna, St-Foy, Quebec). Examples of anti-angiogenic inhibitors that act by blocking the function of endothelial cell-extracellular matrix adhesion molecules and which may be administered in combination with the compositions of the invention include, but are not limited to, EMD-121974 (Merck KcgaA Darmstadt, Germany) and Vitaxin (Ixsys, La Jolla, CA/Medimmune, Gaithersburg, MD). Examples of anti-angiogenic agents that act by directly antagonizing or inhibiting angiogenesis inducers and which may be administered in combination with the compositions of the invention include, but are not limited to, Angiozyme (Ribozyme, Boulder, CO), Anti-VEGF antibody (Genentech, S. San Francisco, CA), PTK-787/ZK-225846 (Novartis,

Basel, Switzerland), SU-101 (Sugen, S. San Francisco, CA), SU-5416 (Sugen/ Pharmacia Upjohn, Bridgewater, NJ), and SU-6668 (Sugen). Other anti-angiogenic agents act to indirectly inhibit angiogenesis. Examples of indirect inhibitors of angiogenesis which may be administered in combination with the compositions of the invention include, but are not limited to, IM-862 (Cytran, Kirkland, WA), Interferon-alpha, IL-12 (Roche, Nutley, NJ), and Pentosan polysulfate (Georgetown University, Washington, DC).

[0615] In particular embodiments, the use of compositions of the invention in combination with anti-angiogenic agents is contemplated for the treatment, prevention, and/or amelioration of an autoimmune disease, such as for example, an autoimmune disease described herein.

[0616] In a particular embodiment, the use of compositions of the invention in combination with anti-angiogenic agents is contemplated for the treatment, prevention, and/or amelioration of arthritis. In a more particular embodiment, the use of compositions of the invention in combination with anti-angiogenic agents is contemplated for the treatment, prevention, and/or amelioration of rheumatoid arthritis.

[0617] In another embodiment, the polynucleotides encoding a polypeptide of the present invention are administered in combination with an angiogenic protein, or polynucleotides encoding an angiogenic protein. Examples of angiogenic proteins that may be administered with the compositions of the invention include, but are not limited to, acidic and basic fibroblast growth factors, VEGF-1, VEGF-2, VEGF-3, epidermal growth factor alpha and beta, platelet-derived endothelial cell growth factor, platelet-derived growth factor, tumor necrosis factor alpha, hepatocyte growth factor, insulin-like growth factor, colony stimulating factor, macrophage colony stimulating factor, granulocyte/macrophage colony stimulating factor, and nitric oxide synthase.

[0618] In additional embodiments, compositions of the invention are administered in combination with a chemotherapeutic agent. Chemotherapeutic agents that may be administered with the Therapeutics of the invention include, but are not limited to alkylating agents such as nitrogen mustards (for example, Mechlorethamine, cyclophosphamide, Cyclophosphamide Ifosfamide, Melphalan (L-sarcolysin), and Chlorambucil), ethylenimines and methylmelamines (for example, Hexamethylmelamine and Thiotepa), alkyl sulfonates (for example, Busulfan), nitrosoureas (for example, Carmustine (BCNU), Lomustine (CCNU), Semustine (methyl-CCNU), and Streptozocin

(streptozotocin)), triazenes (for example, Dacarbazine (DTIC; dimethyltriazenoimidazolecarboxamide)), folic acid analogs (for example, Methotrexate (amethopterin)), pyrimidine analogs (for example, Fluorouracil (5-fluorouracil; 5-FU), Floxuridine (fluorodeoxyuridine; FudR), and Cytarabine (cytosine arabinoside)), purine analogs and related inhibitors (for example, Mercaptopurine (6-mercaptopurine; 6-MP), Thioguanine (6-thioguanine; TG), and Pentostatin (2'-deoxycoformycin)), vinca alkaloids (for example, Vinblastine (VLB, vinblastine sulfate)) and Vincristine (vincristine sulfate)), epipodophyllotoxins (for example, Etoposide and Teniposide), antibiotics (for example, Dactinomycin (actinomycin D), Daunorubicin (daunomycin; rubidomycin), Doxorubicin, Bleomycin, Plicamycin (mithramycin), and Mitomycin (mitomycin C), enzymes (for example, L-Asparaginase), biological response modifiers (for example, Interferon-alpha and interferon-alpha-2b), platinum coordination compounds (for example, Cisplatin (cis-DDP) and Carboplatin), anthracenedione (Mitoxantrone), substituted ureas (for example, Hydroxyurea), methylhydrazine derivatives (for example, Procarbazine (N-methylhydrazine; MIH), adrenocorticosteroids (for example, Prednisone), progestins (for example, Hydroxyprogesterone caproate, Medroxyprogesterone, Medroxyprogesterone acetate, and Megestrol acetate), estrogens (for example, Diethylstilbestrol (DES), Diethylstilbestrol diphosphate, Estradiol, and Ethinyl estradiol), antiestrogens (for example, Tamoxifen), androgens (Testosterone propionate, and Fluoxymesterone), antiandrogens (for example, Flutamide), gonadotropin-releasing hormone analogs (for example, Leuprolide), other hormones and hormone analogs (for example, methyltestosterone, estramustine, estramustine phosphate sodium, chlorotrianisene, and testolactone), and others (for example, dicarbazine, glutamic acid, and mitotane).

[0619] In one embodiment, the compositions of the invention are administered in combination with one or more of the following drugs: infliximab (also known as Remicade™ Centocor, Inc.), Trocade (Roche, RO-32-3555), Leflunomide (also known as Arava™ from Hoechst Marion Roussel), Kineret™ (an IL-1 Receptor antagonist also known as Anakinra from Amgen, Inc.)

[0620] In a specific embodiment, compositions of the invention are administered in combination with CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) or combination of one or more of the components of CHOP. In one embodiment, the compositions of the invention are administered in combination with anti-CD20 antibodies,

human monoclonal anti-CD20 antibodies. In another embodiment, the compositions of the invention are administered in combination with anti-CD20 antibodies and CHOP, or anti-CD20 antibodies and any combination of one or more of the components of CHOP, particularly cyclophosphamide and/or prednisone. In a specific embodiment, compositions of the invention are administered in combination with Rituximab. In a further embodiment, compositions of the invention are administered with Rituximab and CHOP, or Rituximab and any combination of one or more of the components of CHOP, particularly cyclophosphamide and/or prednisone. In a specific embodiment, compositions of the invention are administered in combination with tositumomab. In a further embodiment, compositions of the invention are administered with tositumomab and CHOP, or tositumomab and any combination of one or more of the components of CHOP, particularly cyclophosphamide and/or prednisone. The anti-CD20 antibodies may optionally be associated with radioisotopes, toxins or cytotoxic prodrugs.

[0621] In another specific embodiment, the compositions of the invention are administered in combination Zevalin™. In a further embodiment, compositions of the invention are administered with Zevalin™ and CHOP, or Zevalin™ and any combination of one or more of the components of CHOP, particularly cyclophosphamide and/or prednisone. Zevalin™ may be associated with one or more radioisotopes. Particularly preferred isotopes are ⁹⁰Y and ¹¹¹In.

[0622] In an additional embodiment, the Therapeutics of the invention are administered in combination with cytokines. Cytokines that may be administered with the Therapeutics of the invention include, but are not limited to, IL2, IL3, IL4, IL5, IL6, IL7, IL10, IL12, IL13, IL15, anti-CD40, CD40L, IFN-gamma and TNF-alpha. In another embodiment, Therapeutics of the invention may be administered with any interleukin, including, but not limited to, IL-1alpha, IL-1beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, and IL-21.

[0623] In one embodiment, the Therapeutics of the invention are administered in combination with members of the TNF family. TNF, TNF-related or TNF-like molecules that may be administered with the Therapeutics of the invention include, but are not limited to, soluble forms of TNF-alpha, lymphotoxin-alpha (LT-alpha, also known as TNF-beta), LT-beta (found in complex heterotrimer LT-alpha2-beta), OPGL, FasL, CD27L, CD30L, CD40L, 4-1BBL, DcR3, OX40L, TNF-gamma (International Publication

No. WO 96/14328), AIM-I (International Publication No. WO 97/33899), endokine-alpha (International Publication No. WO 98/07880), OPG, and neutrokin-alpha (International Publication No. WO 98/18921, OX40, and nerve growth factor (NGF), and soluble forms of Fas, CD30, CD27, CD40 and 4-IBB, TR2 (International Publication No. WO 96/34095), DR3 (International Publication No. WO 97/33904), DR4 (International Publication No. WO 98/32856), TR5 (International Publication No. WO 98/30693), TRANK, TR9 (International Publication No. WO 98/56892), TR10 (International Publication No. WO 98/54202), 312C2 (International Publication No. WO 98/06842), and TR12, and soluble forms CD154, CD70, and CD153.

[0624] In an additional embodiment, the Therapeutics of the invention are administered in combination with angiogenic proteins. Angiogenic proteins that may be administered with the Therapeutics of the invention include, but are not limited to, Glioma Derived Growth Factor (GDGF), as disclosed in European Patent Number EP-399816; Platelet Derived Growth Factor-A (PDGF-A), as disclosed in European Patent Number EP-682110; Platelet Derived Growth Factor-B (PDGF-B), as disclosed in European Patent Number EP-282317; Placental Growth Factor (PlGF), as disclosed in International Publication Number WO 92/06194; Placental Growth Factor-2 (PlGF-2), as disclosed in Hauser et al., Growth Factors, 4:259-268 (1993); Vascular Endothelial Growth Factor (VEGF), as disclosed in International Publication Number WO 90/13649; Vascular Endothelial Growth Factor-A (VEGF-A), as disclosed in European Patent Number EP-506477; Vascular Endothelial Growth Factor-2 (VEGF-2), as disclosed in International Publication Number WO 96/39515; Vascular Endothelial Growth Factor B (VEGF-3); Vascular Endothelial Growth Factor B-186 (VEGF-B186), as disclosed in International Publication Number WO 96/26736; Vascular Endothelial Growth Factor-D (VEGF-D), as disclosed in International Publication Number WO 98/02543; Vascular Endothelial Growth Factor-D (VEGF-D), as disclosed in International Publication Number WO 98/07832; and Vascular Endothelial Growth Factor-E (VEGF-E), as disclosed in German Patent Number DE19639601. The above mentioned references are herein incorporated by reference in their entireties.

[0625] In an additional embodiment, the Therapeutics of the invention are administered in combination with Fibroblast Growth Factors. Fibroblast Growth Factors that may be administered with the Therapeutics of the invention include, but are not limited to, FGF-1,

FGF-2, FGF-3, FGF-4, FGF-5, FGF-6, FGF-7, FGF-8, FGF-9, FGF-10, FGF-11, FGF-12, FGF-13, FGF-14, and FGF-15.

[0626] In an additional embodiment, the Therapeutics of the invention are administered in combination with hematopoietic growth factors. Hematopoietic growth factors that may be administered with the Therapeutics of the invention include, but are not limited to, granulocyte macrophage colony stimulating factor (GM-CSF) (sargramostim, LEUKINE™, PROKINE™), granulocyte colony stimulating factor (G-CSF) (filgrastim, NEUPOGEN™), macrophage colony stimulating factor (M-CSF, CSF-1) erythropoietin (epoetin alfa, EPOGEN™, PROCRIT™), stem cell factor (SCF, c-kit ligand, steel factor), megakaryocyte colony stimulating factor, PIXY321 (a GMCSF/IL-3 fusion protein), interleukins, especially any one or more of IL-1 through IL-12, interferon-gamma, or thrombopoietin.

[0627] In certain embodiments, Therapeutics of the present invention are administered in combination with adrenergic blockers, such as, for example, acebutolol, atenolol, betaxolol, bisoprolol, carteolol, labetalol, metoprolol, nadolol, oxprenolol, penbutolol, pindolol, propranolol, sotalol, and timolol.

[0628] In another embodiment, the Therapeutics of the invention are administered in combination with an antiarrhythmic drug (e.g., adenosine, amidoarone, bretylium, digitalis, digoxin, digitoxin, diltiazem, disopyramide, esmolol, flecainide, lidocaine, mexiletine, moricizine, phenytoin, procainamide, N-acetyl procainamide, propafenone, propranolol, quinidine, sotalol, tocainide, and verapamil).

[0629] In another embodiment, the Therapeutics of the invention are administered in combination with diuretic agents, such as carbonic anhydrase-inhibiting agents (e.g., acetazolamide, dichlorphenamide, and methazolamide), osmotic diuretics (e.g., glycerin, isosorbide, mannitol, and urea), diuretics that inhibit $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ symport (e.g., furosemide, bumetanide, azosemide, piretanide, tripamide, ethacrynic acid, muzolimine, and torsemide), thiazide and thiazide-like diuretics (e.g., bendroflumethiazide, benzthiazide, chlorothiazide, hydrochlorothiazide, hydroflumethiazide, methyclothiazide, polythiazide, trichormethiazide, chlorthalidone, indapamide, metolazone, and quinethazone), potassium sparing diuretics (e.g., amiloride and triamterene), and mineralcorticoid receptor antagonists (e.g., spironolactone, canrenone, and potassium canrenoate).

[0630] In one embodiment, the Therapeutics of the invention are administered in combination with treatments for endocrine and/or hormone imbalance disorders. Treatments for endocrine and/or hormone imbalance disorders include, but are not limited to, ^{127}I , radioactive isotopes of iodine such as ^{131}I and ^{123}I ; recombinant growth hormone, such as HUMATROPE™ (recombinant somatropin); growth hormone analogs such as PROTROPIN™ (somatrem); dopamine agonists such as PARLODEL™ (bromocriptine); somatostatin analogs such as SANDOSTATIN™ (octreotide); gonadotropin preparations such as PREGNYL™, A.P.L.™ and PROFASI™ (chorionic gonadotropin (CG)), PERGONAL™ (menotropins), and METRODIN™ (urofollitropin (uFSH)); synthetic human gonadotropin releasing hormone preparations such as FACTREL™ and LUTREPULSE™ (gonadorelin hydrochloride); synthetic gonadotropin agonists such as LUPRON™ (leuprolide acetate), SUPPRELIN™ (histrelin acetate), SYNAREL™ (nafarelin acetate), and ZOLADEX™ (goserelin acetate); synthetic preparations of thyrotropin-releasing hormone such as RELEFACT TRH™ and THYPINONE™ (protirelin); recombinant human TSH such as THYROGEN™; synthetic preparations of the sodium salts of the natural isomers of thyroid hormones such as L-T₄™, SYNTHROID™ and LEVOTHROID™ (levothyroxine sodium), L-T₃™, CYTOMEL™ and TRIOSTAT™ (liothyroine sodium), and THYROLAR™ (liotrix); antithyroid compounds such as 6-*n*-propylthiouracil (propylthiouracil), 1-methyl-2-mercaptoimidazole and TAPAZOLE™ (methimazole), NEO-MERCAZOLE™ (carbimazole); beta-adrenergic receptor antagonists such as propranolol and esmolol; Ca²⁺ channel blockers; dexamethasone and iodinated radiological contrast agents such as TELEPAQUE™ (iopanoic acid) and ORAGRAFIN™ (sodium ipodate).

[0631] Additional treatments for endocrine and/or hormone imbalance disorders include, but are not limited to, estrogens or conjugated estrogens such as ESTRACE™ (estradiol), ESTINYL™ (ethinyl estradiol), PREMARIN™, ESTRATAB™, ORTHO-EST™, OGEN™ and estropipate (estrone), ESTROVIS™ (quinestrol), ESTRADERM™ (estradiol), DELESTROGEN™ and VALERGEN™ (estradiol valerate), DEPO-ESTRADIOL CYPIONATE™ and ESTROJECT LA™ (estradiol cypionate); antiestrogens such as NOLVADEX™ (tamoxifen), SEROPHENE™ and CLOMID™ (clomiphene); progestins such as DURALUTIN™ (hydroxyprogesterone caproate), MPA™ and DEPO-

PROVERA™ (medroxyprogesterone acetate), PROVERA™ and CYCRIN™ (MPA), MEGACE™ (megestrol acetate), NORLUTIN™ (norethindrone), and NORLUTATE™ and AYGESTIN™ (norethindrone acetate); progesterone implants such as NORPLANT SYSTEM™ (subdermal implants of norgestrel); antiprogestins such as RU 486™ (mifepristone); hormonal contraceptives such as ENOVID™ (norethynodrel plus mestranol), PROGESTASERT™ (intrauterine device that releases progesterone), LOESTRIN™, BREVICON™, MODICON™, GENORA™, NELONA™, NORINYL™, OVACON-35™ and OVACON-50™ (ethinyl estradiol/norethindrone), LEVLEN™, NORDETTE™, TRI-LEVLEN™ and TRIPHASIL-21™ (ethinyl estradiol/levonorgestrel) LO/OVRAL™ and OVRAL™ (ethinyl estradiol/norgestrel), DEMULEN™ (ethinyl estradiol/ethynodiol diacetate), NORINYL™, ORTHO-NOVUM™, NORETHIN™, GENORA™, and NELOVA™ (norethindrone/mestranol), DESOGEN™ and ORTHO-CEPT™ (ethinyl estradiol/desogestrel), ORTHO-CYCLEN™ and ORTHO-TRICYCLEN™ (ethinyl estradiol/norgestimate), MICRONOR™ and NOR-QD™ (norethindrone), and OVRETTE™ (norgestrel).

[0632] Additional treatments for endocrine and/or hormone imbalance disorders include, but are not limited to, testosterone esters such as methenolone acetate and testosterone undecanoate; parenteral and oral androgens such as TESTOJECT-50™ (testosterone), TESTEX™ (testosterone propionate), DELATESTRYL™ (testosterone enanthate), DEPO-TESTOSTERONE™ (testosterone cypionate), DANOCRINE™ (danazol), HALOTESTIN™ (fluoxymesterone), ORETON METHYL™, TESTRED™ and VIRILON™ (methyltestosterone), and OXANDRIN™ (oxandrolone); testosterone transdermal systems such as TESTODERM™; androgen receptor antagonist and 5-alpha-reductase inhibitors such as ANDROCUR™ (cyproterone acetate), EULEXIN™ (flutamide), and PROSCAR™ (finasteride); adrenocorticotrophic hormone preparations such as CORTROSYN™ (cosyntropin); adrenocortical steroids and their synthetic analogs such as ACLOVATE™ (alclometasone dipropionate), CYCLOCORT™ (amcinonide), BECLOVENT™ and VANCERIL™ (beclomethasone dipropionate), CELESTONE™ (betamethasone), BENISONE™ and UTICORT™ (betamethasone benzoate), DIPROSONE™ (betamethasone dipropionate), CELESTONE PHOSPHATE™ (betamethasone sodium phosphate), CELESTONE SOLUSPAN™ (betamethasone sodium

phosphate and acetate), BETA-VAL™ and VALISONE™ (betamethasone valerate), TEMOVATE™ (clobetasol propionate), CLODERM™ (clocortolone pivalate), CORTEF™ and HYDROCORTONE™ (cortisol (hydrocortisone)), HYDROCORTONE ACETATE™ (cortisol (hydrocortisone) acetate), LOCOID™ (cortisol (hydrocortisone) butyrate), HYDROCORTONE PHOSPHATE™ (cortisol (hydrocortisone) sodium phosphate), A-HYDROCORT™ and SOLU CORTEF™ (cortisol (hydrocortisone) sodium succinate), WESTCORT™ (cortisol (hydrocortisone) valerate), CORTISONE ACETATE™ (cortisone acetate), DESOWEN™ and TRIDESILON™ (desonide), TOPICORT™ (desoximetasone), DECADRON™ (dexamethasone), DECADRON LA™ (dexamethasone acetate), DECADRON PHOSPHATE™ and HEXADROL PHOSPHATE™ (dexamethasone sodium phosphate), FLORONE™ and MAXIFLOR™ (diflorasone diacetate), FLORINEF ACETATE™ (fludrocortisone acetate), AEROBID™ and NASALIDE™ (flunisolide), FLUONID™ and SYNALAR™ (fluocinolone acetonide), LIDEX™ (fluocinonide), FLUOR-OP™ and FML™ (fluorometholone), CORDRAN™ (flurandrenolide), HALOG™ (halcinonide), HMS LIZUIFILM™ (medrysone), MEDROL™ (methylprednisolone), DEPO-MEDROL™ and MEDROL ACETATE™ (methylprednisone acetate), A-METHAPRED™ and SOLUMEDROL™ (methylprednisolone sodium succinate), ELOCON™ (mometasone furoate), HALDRONE™ (paramethasone acetate), DELTA-CORTEF™ (prednisolone), ECONOPRED™ (prednisolone acetate), HYDELTRASOL™ (prednisolone sodium phosphate), HYDELTRA-T.B.A™ (prednisolone tebutate), DELTASONE™ (prednisone), ARISTOCORT™ and KENACORT™ (triamcinolone), KENALOG™ (triamcinolone acetonide), ARISTOCORT™ and KENACORT DIACETATE™ (triamcinolone diacetate), and ARISTOSPAN™ (triamcinolone hexacetonide); inhibitors of biosynthesis and action of adrenocortical steroids such as CYTADREN™ (aminoglutethimide), NIZORAL™ (ketoconazole), MODRASTANE™ (trilostane), and METOPIRONE™ (metyrapone);

[0633] Additional treatments for endocrine and/or hormone imbalance disorders include, but are not limited to bovine, porcine or human insulin or mixtures thereof; insulin analogs; recombinant human insulin such as HUMULIN™ and NOVOLIN™; oral hypoglycemic agents such as ORAMIDE™ and ORINASE™ (tolbutamide), DIABINESE™ (chlorpropamide), TOLAMIDE™ and TOLINASE™ (tolazamide),

DYMELOR™ (acetohexamide), glibenclamide, MICRONASE™, DIBETA™ and GLYNASE™ (glyburide), GLUCOTROL™ (glipizide), and DIAMICRON™ (gliclazide), GLUCOPHAGE™ (metformin), PRECOSE™ (acarbose), AMARYL™ (glimepiride), and ciglitazone; thiazolidinediones (TZDs) such as rosiglitazone, AVANDIA™ (rosiglitazone maleate) ACTOS™ (pioglitazone), and troglitazone; alpha-glucosidase inhibitors; bovine or porcine glucagon; somatostatins such as SANDOSTATIN™ (octreotide); and diazoxides such as PROGLYCEM™ (diazoxide). In still other embodiments, Therapeutics of the invention are administered in combination with one or more of the following: a biguanide antidiabetic agent, a glitazone antidiabetic agent, and a sulfonylurea antidiabetic agent.

[0634] In one embodiment, the Therapeutics of the invention are administered in combination with treatments for uterine motility disorders. Treatments for uterine motility disorders include, but are not limited to, estrogen drugs such as conjugated estrogens (e.g., PREMARIN® and ESTRATAB®), estradiols (e.g., CLIMARA® and ALORA®), estropipate, and chlorotrianisene; progestin drugs (e.g., AMEN® (medroxyprogesterone), MICRONOR® (norethidrone acetate), PROMETRIUM® progesterone, and megestrol acetate); and estrogen/progesterone combination therapies such as, for example, conjugated estrogens/medroxyprogesterone (e.g., PREMPRO™ and PREMPHASE®) and norethindrone acetate/ethinyl estradiol (e.g., FEMHRT™).

[0635] In an additional embodiment, the Therapeutics of the invention are administered in combination with drugs effective in treating iron deficiency and hypochromic anemias, including but not limited to, ferrous sulfate (iron sulfate, FEOSOL™), ferrous fumarate (e.g., FEOSTAT™), ferrous gluconate (e.g., FERGON™), polysaccharide-iron complex (e.g., NIFEREX™), iron dextran injection (e.g., INFED™), cupric sulfate, pyroxidine, riboflavin, Vitamin B₁₂, cyancobalamin injection (e.g., REDISOL™, RUBRAMIN PC™), hydroxocobalamin, folic acid (e.g., FOLVITE™), leucovorin (folinic acid, 5-CHOH4PteGlu, citrovorum factor) or WELLCOVORIN (Calcium salt of leucovorin), transferrin or ferritin.

[0636] In certain embodiments, the Therapeutics of the invention are administered in combination with agents used to treat psychiatric disorders. Psychiatric drugs that may be administered with the Therapeutics of the invention include, but are not limited to,

antipsychotic agents (e.g., chlorpromazine, chlorprothixene, clozapine, fluphenazine, haloperidol, loxapine, mesoridazine, molindone, olanzapine, perphenazine, pimozide, quetiapine, risperidone, thioridazine, thiothixene, trifluoperazine, and triflupromazine), antimanic agents (e.g., carbamazepine, divalproex sodium, lithium carbonate, and lithium citrate), antidepressants (e.g., amitriptyline, amoxapine, bupropion, citalopram, clomipramine, desipramine, doxepin, fluvoxamine, fluoxetine, imipramine, isocarboxazid, maprotiline, mirtazapine, nefazodone, nortriptyline, paroxetine, phenelzine, protriptyline, sertraline, tranylcypromine, trazodone, trimipramine, and venlafaxine), antianxiety agents (e.g., alprazolam, buspirone, chlordiazepoxide, clorazepate, diazepam, halazepam, lorazepam, oxazepam, and prazepam), and stimulants (e.g., d-amphetamine, methylphenidate, and pemoline).

[0637] In other embodiments, the Therapeutics of the invention are administered in combination with agents used to treat neurological disorders. Neurological agents that may be administered with the Therapeutics of the invention include, but are not limited to, antiepileptic agents (e.g., carbamazepine, clonazepam, ethosuximide, phenobarbital, phenytoin, primidone, valproic acid, divalproex sodium, felbamate, gabapentin, lamotrigine, levetiracetam, oxcarbazepine, tiagabine, topiramate, zonisamide, diazepam, lorazepam, and clonazepam), antiparkinsonian agents (e.g., levodopa/carbidopa, selegiline, amantidine, bromocriptine, pergolide, ropinirole, pramipexole, benztropine; biperiden; ethopropazine; procyclidine; trihexyphenidyl, tolcapone), and ALS therapeutics (e.g. riluzole).

[0638] In another embodiment, Therapeutics of the invention are administered in combination with vasodilating agents and/or calcium channel blocking agents. Vasodilating agents that may be administered with the Therapeutics of the invention include, but are not limited to, Angiotensin Converting Enzyme (ACE) inhibitors (e.g., papaverine, isoxsuprine, benazepril, captopril, cilazapril, enalapril, enalaprilat, fosinopril, lisinopril, moexipril, perindopril, quinapril, ramipril, spirapril,trandolapril, and nylidrin), and nitrates (e.g., isosorbide dinitrate, isosorbide mononitrate, and nitroglycerin). Examples of calcium channel blocking agents that may be administered in combination with the Therapeutics of the invention include, but are not limited to amlodipine, bepridil, diltiazem, felodipine, flunarizine, isradipine, nicardipine, nifedipine, nimodipine, and verapamil.

[0639] In certain embodiments, the Therapeutics of the invention are administered in combination with treatments for gastrointestinal disorders. Treatments for gastrointestinal disorders that may be administered with the Therapeutic of the invention include, but are not limited to, H₂ histamine receptor antagonists (e.g., TAGAMETTM (cimetidine), ZANTACTM (ranitidine), PEPCIDTM (famotidine), and AXIDTM (nizatidine)); inhibitors of H⁺, K⁺ ATPase (e.g., PREVACIDTM (lansoprazole) and PRILOSECTM (omeprazole)); Bismuth compounds (e.g., PEPTO-BISMOLTM (bismuth subsalicylate) and DE-NOLTM (bismuth subcitrate)); various antacids; sucralfate; prostaglandin analogs (e.g. CYTOTECTM (misoprostol)); muscarinic cholinergic antagonists; laxatives (e.g., surfactant laxatives, stimulant laxatives, saline and osmotic laxatives); antidiarrheal agents (e.g., LOMOTILTM (diphenoxylate), MOTOFENTM (diphenoxin), and IMMIDIUMTM (loperamide hydrochloride)), synthetic analogs of somatostatin such as SANDOSTATINTM (octreotide), antiemetic agents (e.g., ZOFRANTM (ondansetron), KYTRILTM (granisetron hydrochloride), tropisetron, dolasetron, metoclopramide, chlorpromazine, perphenazine, prochlorperazine, promethazine, thiethylperazine, triflupromazine, domperidone, haloperidol, droperidol, trimethobenzamide, dexamethasone, methylprednisolone, dronabinol, and nabilone); D₂ antagonists (e.g., metoclopramide, trimethobenzamide and chlorpromazine); bile salts; chenodeoxycholic acid; ursodeoxycholic acid; and pancreatic enzyme preparations such as pancreatin and pancrelipase.

[0640] In additional embodiments, the Therapeutics of the invention are administered in combination with other therapeutic or prophylactic regimens, such as, for example, radiation therapy.

Example 14: Method of Treating Decreased Levels of Stanniocalcin

[0641] The present invention relates to a method for treating an individual in need of a decreased level of stanniocalcin activity in the body comprising, administering to such an individual a composition comprising a therapeutically effective amount of stanniocalcin antagonist. Preferred antagonists for use in the present invention are stanniocalcin-specific antibodies.

[0642] Moreover, it will be appreciated that conditions caused by a decrease in the standard or normal expression level of stanniocalcin in an individual can be treated by administering stanniocalcin, preferably in the secreted form. Thus, the invention also provides a method of treatment of an individual in need of an increased level of stanniocalcin polypeptide comprising administering to such an individual a pharmaceutical composition comprising an amount of stanniocalcin to increase the activity level of stanniocalcin in such an individual.

[0643] For example, a patient with decreased levels of stanniocalcin polypeptide receives a daily dose 0.1-100 ug/kg of the polypeptide for six consecutive days. Preferably, the polypeptide is in the secreted form. The exact details of the dosing scheme, based on administration and formulation, are provided in Example 24.

Example 15: Method of Treating Increased Levels of Stanniocalcin

[0644] The present invention also relates to a method for treating an individual in need of an increased level of stanniocalcin activity in the body comprising administering to such an individual a composition comprising a therapeutically effective amount of stanniocalcin or an agonist thereof.

[0645] Antisense technology is used to inhibit production of stanniocalcin. This technology is one example of a method of decreasing levels of stanniocalcin polypeptide, preferably a secreted form, due to a variety of etiologies, such as cancer.

[0646] For example, a patient diagnosed with abnormally increased levels of stanniocalcin is administered intravenously antisense polynucleotides at 0.5, 1.0, 1.5, 2.0 and 3.0 mg/kg day for 21 days. This treatment is repeated after a 7-day rest period if the treatment was well tolerated. The formulation of the antisense polynucleotide is provided in Example 24.

Example 16: Method of Treatment Using Gene Therapy - Ex Vivo

[0647] One method of gene therapy transplants fibroblasts, which are capable of expressing stanniocalcin polypeptides, onto a patient. Generally, fibroblasts are obtained

from a subject by skin biopsy. The resulting tissue is placed in tissue-culture medium and separated into small pieces. Small chunks of the tissue are placed on a wet surface of a tissue culture flask, approximately ten pieces are placed in each flask. The flask is turned upside down, closed tight and left at room temperature over night. After 24 hours at room temperature, the flask is inverted and the chunks of tissue remain fixed to the bottom of the flask and fresh media (e.g., Ham's F12 media, with 10% FBS, penicillin and streptomycin) is added. The flasks are then incubated at 37 degree C for approximately one week.

[0648] At this time, fresh media is added and subsequently changed every several days. After an additional two weeks in culture, a monolayer of fibroblasts emerge. The monolayer is trypsinized and scaled into larger flasks.

[0649] pMV-7 (Kirschmeier, P.T. et al., DNA, 7:219-25 (1988)), flanked by the long terminal repeats of the Moloney murine sarcoma virus, is digested with EcoRI and HindIII and subsequently treated with calf intestinal phosphatase. The linear vector is fractionated on agarose gel and purified, using glass beads.

[0650] The cDNA encoding stanniocalcin can be amplified using PCR primers which correspond to the 5' and 3' end sequences respectively as set forth in Example 1. Preferably, the 5' primer contains an EcoRI site and the 3' primer includes a HindIII site. Equal quantities of the Moloney murine sarcoma virus linear backbone and the amplified EcoRI and HindIII fragment are added together, in the presence of T4 DNA ligase. The resulting mixture is maintained under conditions appropriate for ligation of the two fragments. The ligation mixture is then used to transform bacteria HB101, which are then plated onto agar containing kanamycin for the purpose of confirming that the vector contains properly inserted stanniocalcin.

[0651] The amphotropic pA317 or GP+am12 packaging cells are grown in tissue culture to confluent density in Dulbecco's Modified Eagles Medium (DMEM) with 10% calf serum (CS), penicillin and streptomycin. The MSV vector containing the stanniocalcin gene is then added to the media and the packaging cells transduced with the vector. The packaging cells now produce infectious viral particles containing the stanniocalcin gene(the packaging cells are now referred to as producer cells).

[0652] Fresh media is added to the transduced producer cells, and subsequently, the media is harvested from a 10 cm plate of confluent producer cells. The spent media,

containing the infectious viral particles, is filtered through a Millipore filter to remove detached producer cells and this media is then used to infect fibroblast cells. Media is removed from a sub-confluent plate of fibroblasts and quickly replaced with the media from the producer cells. This media is removed and replaced with fresh media. If the titer of virus is high, then virtually all fibroblasts will be infected and no selection is required. If the titer is very low, then it is necessary to use a retroviral vector that has a selectable marker, such as neo or his. Once the fibroblasts have been efficiently infected, the fibroblasts are analyzed to determine whether stanniocalcin protein is produced.

[0653] The engineered fibroblasts are then transplanted onto the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads.

Example 17: Gene Therapy Using Endogenous Stanniocalcin Gene

[0654] Another method of gene therapy according to the present invention involves operably associating the endogenous stanniocalcin sequence with a promoter via homologous recombination as described, for example, in U.S. Patent No. 5,641,670, issued June 24, 1997; International Publication No. WO 96/29411, published September 26, 1996; International Publication No. WO 94/12650, published August 4, 1994; Koller et al., Proc. Natl. Acad. Sci. USA 86:8932-8935 (1989); and Zijlstra et al., Nature 342:435-438 (1989). This method involves the activation of a gene which is present in the target cells, but which is not expressed in the cells, or is expressed at a lower level than desired.

[0655] Polynucleotide constructs are made which contain a promoter and targeting sequences, which are homologous to the 5' non-coding sequence of endogenous stanniocalcin, flanking the promoter. The targeting sequence will be sufficiently near the 5' end of stanniocalcin so the promoter will be operably linked to the endogenous sequence upon homologous recombination. The promoter and the targeting sequences can be amplified using PCR. Preferably, the amplified promoter contains distinct restriction enzyme sites on the 5' and 3' ends. Preferably, the 3' end of the first targeting sequence contains the same restriction enzyme site as the 5' end of the amplified promoter and the 5' end of the second targeting sequence contains the same restriction site as the 3' end of the amplified promoter.

[0656] The amplified promoter and the amplified targeting sequences are digested with the appropriate restriction enzymes and subsequently treated with calf intestinal phosphatase. The digested promoter and digested targeting sequences are added together in the presence of T4 DNA ligase. The resulting mixture is maintained under conditions appropriate for ligation of the two fragments. The construct is size fractionated on an agarose gel then purified by phenol extraction and ethanol precipitation.

[0657] In this Example, the polynucleotide constructs are administered as naked polynucleotides via electroporation. However, the polynucleotide constructs may also be administered with transfection-facilitating agents, such as liposomes, viral sequences, viral particles, precipitating agents, etc. Such methods of delivery are known in the art.

[0658] Once the cells are transfected, homologous recombination will take place which results in the promoter being operably linked to the endogenous stanniocalcin sequence. This results in the expression of stanniocalcin in the cell. Expression may be detected by immunological staining, or any other method known in the art.

[0659] Fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in DMEM + 10% fetal calf serum. Exponentially growing or early stationary phase fibroblasts are trypsinized and rinsed from the plastic surface with nutrient medium. An aliquot of the cell suspension is removed for counting, and the remaining cells are subjected to centrifugation. The supernatant is aspirated and the pellet is resuspended in 5 ml of electroporation buffer (20 mM HEPES pH 7.3, 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂ HPO₄, 6 mM dextrose). The cells are recentrifuged, the supernatant aspirated, and the cells resuspended in electroporation buffer containing 1 mg/ml acetylated bovine serum albumin. The final cell suspension contains approximately 3X10⁶ cells/ml. Electroporation should be performed immediately following resuspension.

[0660] Plasmid DNA is prepared according to standard techniques. For example, to construct a plasmid for targeting to the stanniocalcin locus, plasmid pUC18 (MBI Fermentas, Amherst, NY) is digested with HindIII. The CMV promoter is amplified by PCR with an XbaI site on the 5' end and a BamHI site on the 3'end. Two stanniocalcin non-coding sequences are amplified via PCR: one stanniocalcin non-coding sequence (stanniocalcin fragment 1) is amplified with a HindIII site at the 5' end and an Xba site at the 3'end; the other stanniocalcin non-coding sequence (stanniocalcin fragment 2) is amplified with a BamHI site at the 5'end and a HindIII site at the 3'end. The CMV

promoter and stanniocalcin fragments are digested with the appropriate enzymes (CMV promoter - XbaI and BamHI; stanniocalcin fragment 1 - XbaI; stanniocalcin fragment 2 - BamHI) and ligated together. The resulting ligation product is digested with HindIII, and ligated with the HindIII-digested pUC18 plasmid.

[0661] Plasmid DNA is added to a sterile cuvette with a 0.4 cm electrode gap (Bio-Rad). The final DNA concentration is generally at least 120 $\mu\text{g/ml}$. 0.5 ml of the cell suspension (containing approximately 1.5×10^6 cells) is then added to the cuvette, and the cell suspension and DNA solutions are gently mixed. Electroporation is performed with a Gene-Pulser apparatus (Bio-Rad). Capacitance and voltage are set at 960 μF and 250-300 V, respectively. As voltage increases, cell survival decreases, but the percentage of surviving cells that stably incorporate the introduced DNA into their genome increases dramatically. Given these parameters, a pulse time of approximately 14-20 mSec should be observed.

[0662] Electroporated cells are maintained at room temperature for approximately 5 min, and the contents of the cuvette are then gently removed with a sterile transfer pipette. The cells are added directly to 10 ml of prewarmed nutrient media (DMEM with 15% calf serum) in a 10 cm dish and incubated at 37 degree C. The following day, the media is aspirated and replaced with 10 ml of fresh media and incubated for a further 16-24 hours.

[0663] The engineered fibroblasts are then injected into the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads. The fibroblasts now produce the protein product. The fibroblasts can then be introduced into a patient as described above.

Example 18: Method of Treatment Using Gene Therapy - In Vivo

[0664] Another aspect of the present invention is using in vivo gene therapy methods to treat disorders, diseases and conditions. The gene therapy method relates to the introduction of naked nucleic acid (DNA, RNA, and antisense DNA or RNA) stanniocalcin sequences into an animal to increase or decrease the expression of the stanniocalcin polypeptide. The stanniocalcin polynucleotide may be operatively linked to a promoter or any other genetic elements necessary for the expression of the stanniocalcin

polypeptide by the target tissue. Such gene therapy and delivery techniques and methods are known in the art, see, for example, WO90/11092, WO98/11779; U.S. Patent NO. 5693622, 5705151, 5580859; Tabata H. et al. (1997) *Cardiovasc. Res.* 35(3):470-479, Chao J et al. (1997) *Pharmacol. Res.* 35(6):517-522, Wolff J.A. (1997) *Neuromuscul. Disord.* 7(5):314-318, Schwartz B. et al. (1996) *Gene Ther.* 3(5):405-411, Tsurumi Y. et al. (1996) *Circulation* 94(12):3281-3290 (incorporated herein by reference).

[0665] The stanniocalcin polynucleotide constructs may be delivered by any method that delivers injectable materials to the cells of an animal, such as, injection into the interstitial space of tissues (heart, muscle, skin, lung, liver, intestine and the like). The stanniocalcin polynucleotide constructs can be delivered in a pharmaceutically acceptable liquid or aqueous carrier.

[0666] The term “naked” polynucleotide, DNA or RNA, refers to sequences that are free from any delivery vehicle that acts to assist, promote, or facilitate entry into the cell, including viral sequences, viral particles, liposome formulations, lipofectin or precipitating agents and the like. However, the stanniocalcin polynucleotides may also be delivered in liposome formulations (such as those taught in Felgner P.L. et al. (1995) *Ann. NY Acad. Sci.* 772:126-139 and Abdallah B. et al. (1995) *Biol. Cell* 85(1):1-7) which can be prepared by methods well known to those skilled in the art.

[0667] The stanniocalcin polynucleotide vector constructs used in the gene therapy method are preferably constructs that will not integrate into the host genome nor will they contain sequences that allow for replication. Any strong promoter known to those skilled in the art can be used for driving the expression of DNA. Unlike other gene therapies techniques, one major advantage of introducing naked nucleic acid sequences into target cells is the transitory nature of the polynucleotide synthesis in the cells. Studies have shown that non-replicating DNA sequences can be introduced into cells to provide production of the desired polypeptide for periods of up to six months.

[0668] The stanniocalcin polynucleotide construct can be delivered to the interstitial space of tissues within the an animal, including of muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, and connective tissue. Interstitial space of the tissues comprises the intercellular fluid, mucopolysaccharide matrix among the reticular fibers of organ tissues, elastic fibers in the

walls of vessels or chambers, collagen fibers of fibrous tissues, or that same matrix within connective tissue ensheathing muscle cells or in the lacunae of bone. It is similarly the space occupied by the plasma of the circulation and the lymph fluid of the lymphatic channels. Delivery to the interstitial space of muscle tissue is preferred. They may be conveniently delivered by injection into the tissues comprising these cells. They are preferably delivered to and expressed in persistent, non-dividing cells which are differentiated, although delivery and expression may be achieved in non-differentiated or less completely differentiated cells, such as, for example, stem cells of blood or skin fibroblasts. In vivo muscle cells are particularly competent in their ability to take up and express polynucleotides.

[0669] For the naked stanniocalcin polynucleotide injection, an effective dosage amount of DNA or RNA will be in the range of from about 0.05 g/kg body weight to about 50 mg/kg body weight. Preferably the dosage will be from about 0.005 mg/kg to about 20 mg/kg and more preferably from about 0.05 mg/kg to about 5 mg/kg. Of course, as the artisan of ordinary skill will appreciate, this dosage will vary according to the tissue site of injection. The appropriate and effective dosage of nucleic acid sequence can readily be determined by those of ordinary skill in the art and may depend on the condition being treated and the route of administration. The preferred route of administration is by the parenteral route of injection into the interstitial space of tissues. However, other parenteral routes may also be used, such as, inhalation of an aerosol formulation particularly for delivery to lungs or bronchial tissues, throat or mucous membranes of the nose. In addition, naked stanniocalcin polynucleotide constructs can be delivered to arteries during angioplasty by the catheter used in the procedure.

[0670] The dose response effects of injected stanniocalcin polynucleotide in muscle in vivo is determined as follows. Suitable stanniocalcin template DNA for production of mRNA coding for stanniocalcin polypeptide is prepared in accordance with a standard recombinant DNA methodology. The template DNA, which may be either circular or linear, is either used as naked DNA or complexed with liposomes. The quadriceps muscles of mice are then injected with various amounts of the template DNA.

[0671] Five to six week old female and male Balb/C mice are anesthetized by intraperitoneal injection with 0.3 ml of 2.5% Avertin. A 1.5 cm incision is made on the anterior thigh, and the quadriceps muscle is directly visualized. The stanniocalcin

template DNA is injected in 0.1 ml of carrier in a 1 cc syringe through a 27 gauge needle over one minute, approximately 0.5 cm from the distal insertion site of the muscle into the knee and about 0.2 cm deep. A suture is placed over the injection site for future localization, and the skin is closed with stainless steel clips.

[0672] After an appropriate incubation time (e.g., 7 days) muscle extracts are prepared by excising the entire quadriceps. Every fifth 15 um cross-section of the individual quadriceps muscles is histochemically stained for stanniocalcin protein expression. A time course for stanniocalcin protein expression may be done in a similar fashion except that quadriceps from different mice are harvested at different times. Persistence of stanniocalcin DNA in muscle following injection may be determined by Southern blot analysis after preparing total cellular DNA and HIRT supernatants from injected and control mice. The results of the above experimentation in mice can be use to extrapolate proper dosages and other treatment parameters in humans and other animals using stanniocalcin naked DNA.

Example 19: Stanniocalcin Biological Effects

Astrocyte and Neuronal Assays:

[0673] Recombinant stanniocalcin, expressed in *Escherichia coli* and purified as described above, can be tested for activity in promoting the survival, neurite outgrowth, or phenotypic differentiation of cortical neuronal cells and for inducing the proliferation of glial fibrillary acidic protein immunopositive cells, astrocytes. The selection of cortical cells for the bioassay is based on the prevalent expression of FGF-1 and FGF-2 in cortical structures and on the previously reported enhancement of cortical neuronal survival resulting from FGF-2 treatment. A thymidine incorporation assay, for example, can be used to elucidate stanniocalcin's activity on these cells.

[0674] Moreover, previous reports describing the biological effects of FGF-2 (basic FGF) on cortical or hippocampal neurons *in vitro* have demonstrated increases in both neuron survival and neurite outgrowth (Walicke, P. et al., "Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension." *Proc. Natl. Acad. Sci. USA* 83:3012-3016. (1986), assay herein incorporated by reference

in its entirety). However, reports from experiments done on PC-12 cells suggest that these two responses are not necessarily synonymous and may depend on not only which FGF is being tested but also on which receptor(s) are expressed on the target cells. Using the primary cortical neuronal culture paradigm, the ability of stanniocalcin to induce neurite outgrowth can be compared to the response achieved with FGF-2 using, for example, a thymidine incorporation assay.

Parkinson Models:

[0675] The loss of motor function in Parkinson's disease is attributed to a deficiency of striatal dopamine resulting from the degeneration of the nigrostriatal dopaminergic projection neurons. An animal model for Parkinson's that has been extensively characterized involves the systemic administration of 1-methyl-4 phenyl 1,2,3,6-tetrahydropyridine (MPTP). In the CNS, MPTP is taken-up by astrocytes and catabolized by monoamine oxidase B to 1-methyl-4-phenyl pyridine (MPP⁺) and released. Subsequently, MPP⁺ is actively accumulated in dopaminergic neurons by the high-affinity reuptake transporter for dopamine. MPP⁺ is then concentrated in mitochondria by the electrochemical gradient and selectively inhibits nicotinamide adenine disphosphate: ubiquinone oxidoreductionase (complex I), thereby interfering with electron transport and eventually generating oxygen radicals.

[0676] It has been demonstrated in tissue culture paradigms that FGF-2 (basic FGF) has trophic activity towards nigral dopaminergic neurons (Ferrari et al., Dev. Biol. 1989). Recently, Dr. Unsicker's group has demonstrated that administering FGF-2 in gel foam implants in the striatum results in the near complete protection of nigral dopaminergic neurons from the toxicity associated with MPTP exposure (Otto and Unsicker, J. Neuroscience, 1990).

[0677] Based on the data with FGF-2, stanniocalcin can be evaluated to determine whether it has an action similar to that of FGF-2 in enhancing dopaminergic neuronal survival *in vitro* and it can also be tested *in vivo* for protection of dopaminergic neurons in the striatum from the damage associated with MPTP treatment. The potential effect of stanniocalcin is first examined *in vitro* in a dopaminergic neuronal cell culture paradigm. The cultures are prepared by dissecting the midbrain floor plate from gestation day 14 Wistar rat embryos. The tissue is dissociated with trypsin and seeded at a density of

200,000 cells/cm² on polyorthinine-laminin coated glass coverslips. The cells are maintained in Dulbecco's Modified Eagle's medium and F12 medium containing hormonal supplements (N1). The cultures are fixed with paraformaldehyde after 8 days in vitro and are processed for tyrosine hydroxylase, a specific marker for dopaminergic neurons, immunohistochemical staining. Dissociated cell cultures are prepared from embryonic rats. The culture medium is changed every third day and the factors are also added at that time.

[0678] Since the dopaminergic neurons are isolated from animals at gestation day 14, a developmental time which is past the stage when the dopaminergic precursor cells are proliferating, an increase in the number of tyrosine hydroxylase immunopositive neurons would represent an increase in the number of dopaminergic neurons surviving *in vitro*. Therefore, if stanniocalcin acts to prolong the survival of dopaminergic neurons, it would suggest that stanniocalcin may be involved in Parkinson's Disease.

[0679] The studies described in this example tested activity in stanniocalcin protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of stanniocalcin polynucleotides (e.g., gene therapy), agonists, and/or antagonists of stanniocalcin.

[0680] It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples. Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

[0681] The entire disclosure of each document cited (including patents, patent applications, journal articles, abstracts, laboratory manuals, books, or other disclosures) in the Background of the Invention, Detailed Description, and Examples is hereby incorporated herein by reference.

[0682] Certain Stanniocalcin polynucleotides and polypeptides of the present invention, including antibodies, were disclosed in U.S. Patent No. 5,837,498 and provisional application Serial No. 60/161,740, filed October 27, 1999, as well as in International application number PCT/US00/29432, filed October 26, 2000, the specification and sequence listing of each of which are herein incorporated by reference in their entirety.